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Klotho Is a Novel β -Glucuronidase Capable of Hydrolyzing Steroid β -Glucuronides*

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Klotho mutant mice provide a unique model to analyze mechanisms of aging because their phenotypes resemble those of human aging-associated disorders. The *klotho* gene encodes Klotho, a type I membrane protein that shares sequence similarity with members of the glycosidase family 1. Because Klotho lacks the glutamic acid residues that have been shown to be involved in the catalytic activity of this family of enzymes, the function of this protein was unknown. Here, we have studied the biochemical characteristics of recombinant Klotho. The purified chimeric Klotho-human IgG1 Fc protein (KLFC) was assayed with a series of 4-methylumbelliferyl (4Mu) β -glycosides as potential substrates. An enzymatic activity of Klotho was observed only with 4-methylumbelliferyl β -D-glucuronide in contrast to bovine liver β -glucuronidase, which exhibits a rather wide substrate specificity. Furthermore, the enzymatic activity of KLFC was reduced by the addition of specific inhibitors of β -glucuronidase. A number of natural β -glucuronides were screened by competitive inhibition for KLFC β -glucuronidase. We found that steroid β -glucuronides such as β -estradiol 3- β -D-glucuronide, estrone 3- β -D-glucuronide, and estriol 3- β -D-glucuronide were hydrolyzed by KLFC. The artificial fluorescent substrate and the steroid conjugates share a common phenolic structure. Collectively, these data suggest that Klotho functions as a novel β -glucuronidase and that steroid β -glucuronides are potential candidates for the natural substrate(s) of Klotho.

klotho mutant mice (1, 2) were originally described as a model of human aging-associated disorders because the phenotype observed in this mutant includes arteriosclerosis, ectopic calcification, emphysema, and osteoporosis. The responsible gene, *klotho* (1), encodes a type I membrane glycoprotein of 130 kDa. Expression of this gene is seen in renal tubule cells, parathyroid secretory cells, and epithelial cells of the choroid plexus. The extracellular domain of Klotho contains two inter-

nal repeats (termed mKL1 and mKL2) that display significant similarity to family 1 β -glycosidases (3–5).

Glycosidases are the enzymes that hydrolyze the glycosidic bond between two carbohydrates or between carbohydrate and non-carbohydrate moieties (6, 7). Glycosidases have been classified into about 90 families on the basis of amino acid similarities ((3–5) carbohydrate-active enzymes (CAZY) data base at afmb.cnrs-mrs.fr/~cazy/CAZY/index.html), and they participate in essential steps of synthesis and degradation of oligosaccharides and polysaccharides (8), which are involved in pathogen defense systems (9, 10), detoxification (11, 12), control of signal transduction (13), and modification of hormone (14) among others. In humans, inheritable deficiencies of glycosidases are known to induce a variety of impairments such as lysosomal storage diseases (15), Gaucher's and Krabbe's diseases, and lactose intolerance (16–18). Glycosidase family 1 groups together enzymes of varying substrate specificity (3–5), such as β -glucosidase, β -galactosidase, 6-phospho- β -glucosidase, 6-phospho- β -galactosidase, myrosinase, and lactase-phlorizin hydrolase (LPH).¹ These enzymes are widely distributed throughout bacteria, archaea, and eukaryotes. Family 1 glycosidases operate with a molecular mechanism leading to overall retention of the anomeric configuration and involving the formation and breakdown of a covalent glycosyl enzyme intermediate. Two Glu residues are directly involved in this catalytic mechanism, one acting as a nucleophile and the other as an acid/base (19, 20). Intriguingly, unlike other members of β -glycosidase family 1, Klotho does not possess these two catalytic glutamic acids. In the KL1 region, the nucleophilic Glu residue is present, but the acid/base Glu residue is replaced by an Asn residue. Inversely, in the KL2 region, the acid/base Glu residue is present, but the nucleophile is replaced by Ala and Ser residues in mouse and human Klotho, respectively (1). Together with Klotho, other proteins form a distinct subfamily in glycosidase family 1. First, β -Klotho (β KL) (21), a type I membrane protein with 41.2% similarity to Klotho, contains two internal repeats highly homologous to those of family 1 glycosidases. Like in Klotho, the first repeat lacks the catalytic acid/base, whereas the second repeat lacks the nucleophile. Another protein, Klotho-LPH-related (KLPH) (22), which is also a type I membrane protein, has a single extracellular domain with 37.5% similarity to the KL1 domain of the mouse Klotho proteins, and this domain lacks the acid/base catalytic

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¹ The abbreviations used are: LPH, lactase-phlorizin hydrolase; CHO, Chinese hamster ovary; Fc, crystallizable fragment; GlcU, glucuronide; HPLC, high performance liquid chromatography; IdoUA, iduronic acid; KL, Klotho; 4Mu, 4-methylumbelliferyl.

residue. LPH (23), a major glycosidase contributing to the metabolism of dietary lactose in mammals, is a membrane-bound enzyme that acts extracellularly. The mature form of this enzyme is composed of two internal repeats (LPH3 and LPH4, each belonging to glycosidase family 1) and a transmembrane domain with a short cytoplasmic domain at the carboxyl terminus. The overall structure of the mature form of LPH resembles that of Klotho, and each of the Klotho internal repeats exhibits 36–42% similarity to those of LPH, respectively. Unlike Klotho, however, each of the LPH repeats possesses the two catalytic glutamic acids of glycosidase family 1. In contrast, it should be noted that myrosinase (9, 10), a plant homolog with 28–32% similarity to Klotho, has *S*-glycosidase enzymatic activity, even though it lacks the glutamic acid that serves as a general acid/general base catalyst. For myrosinase, it has been shown that the acid/base residue is not necessary in the first step of catalysis because of the excellent leaving group ability of the aglycone of the natural substrates (glucosinolates). For the second step, Burmeister and colleagues (24) have shown by high resolution x-ray crystallography that ascorbate is a cofactor for myrosinase and substitutes for the function of the catalytic base.

The deficiency of the Klotho proteins leads to impaired calcium homeostasis and enhancement of the expression of the 1 α -hydroxylase gene, the rate-limiting enzyme in 1,25-(OH)₂-vitamin D synthesis, and results in persistently high serum levels of 1,25-(OH)₂-vitamin D in *kl*^{-/-} mice (25, 26). This deterioration in the vitamin D endocrine system may participate in many of the phenotypes in *kl*^{-/-} mice via toxicity resulting from increased levels of 1,25-(OH)₂-vitamin D and calcium (26). Notably, alleviation of many of the phenotypes is observed despite Klotho protein deficiency, when serum concentrations of calcium and 1,25-(OH)₂-vitamin D are restored to normal levels (26). It is possible that Klotho is in the negative regulatory signaling circuit of vitamin D synthesis.

As to how Klotho regulates calcium homeostasis, one of the most obvious hypotheses is that Klotho acts as an enzyme because it displays significant similarity to members of β -glycosidase family 1. If this is so, one may hypothesize that the putative substrate(s) is processed to an active form by the enzymatic action of Klotho, and thus Klotho action could be achieved via the action of the undefined bioactive substrate.

We have examined the biochemical characteristics of Klotho to resolve its putative enzymatic activity. An unexpected synthetic substrate, 4-methylumbelliferyl β -D-glucuronide, was found to be hydrolyzed by Klotho at a slow reaction rate but in a fairly specific manner compared with other family members. We then screened natural substrates with similar structures and found that steroid β -glucuronides were hydrolyzed by Klotho. Here we report that Klotho likely functions as an enzyme in a unique fashion paralleling that of β -glucuronidase.

EXPERIMENTAL PROCEDURES

Expression Constructs—We designed a fusion protein that consists of the extracellular domain of mouse Klotho (amino acids 1–958) fused with human immunoglobulin Fc region at its carboxyl terminus. The expression vector was constructed as follows. Translational initiation sequences and restriction sites were introduced to the 5'-region of mouse *klotho* cDNA via PCR amplification using the primers 5-aaagaatcgccaccatgctagcccgccg-3 (mKL-S1; conjugated EcoRI site and an artificial Kozak initiation) and 5-catggctgaattctccagtcag-3 (mKL-AS1). To add restriction enzyme recognition sites into the 3'-region, primers 5-catggctgaattctccagtcag-3 (mKL-S2) and 5-cccagtggtccagatccaggaagc-3 (mKL-AS2) were used. These PCR fragments were ligated with the PstI-FspI fragment of *klotho* cDNA and cloned into pSP72, giving PSP72-KL full 3. Then this was digested with EcoRI and BamHI and ligated with the BamHI-XbaI fragment of the human IgG1 Fc gene (27), inserted into expression vector pYT103 to give KLFc/pYT103. LPH

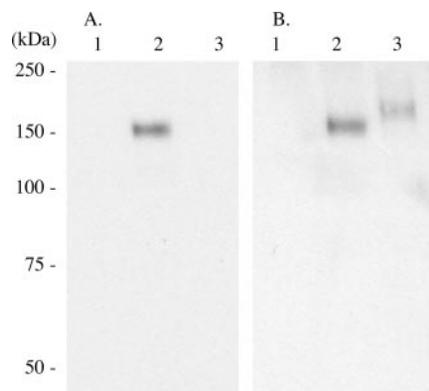


FIG. 1. Western blot analysis of fusion proteins used for the enzyme assay. Conditioned media from CHO cells that were transfected with expression constructs for mock control (*lane 1*), Klotho fused with human IgG1 Fc (KLFc) (*lane 2*), or LPH fused with human IgG1 LPHFc (*lane 3*) were analyzed by immunoblotting. Bands were detected with anti-Klotho (A) and anti-Fc tag (B) antibodies. The positions of molecular mass standards are indicated on the left.

(domains 3 and 4)-Fc fusion protein (LPHFc), used as a positive control, was designed similarly. Restriction sites were introduced to rat LPH cDNA by PCR amplification with primers 5-tggcttcggaggatcttgaac-3 (LPH-S1) and 5-aaactcgagatctgtctgtctctgctgcatgccc-3 (LPH-AS1; conjugated BglI site at the 3'-region). This PCR fragment was ligated with BamHI-XbaI fragment of human IgG Fc gene and cloned into the pcDNA3 expression vector to give LPHFc/pcDNA3.

Cell Transfection—CHO cells were cultured in minimum essential medium- α (Sigma) containing 1% (v/v) fetal bovine serum, 100 units/ml penicillin, 0.0001% (w/v) streptomycin, and 2 mM L-glutamine (Sigma). To establish CHO clones stably expressing the chimeric proteins, 4 μ g of plasmids were introduced into 10⁷ cells using LipofectAMINE Plus reagent (Invitrogen). For KLFc, stable transfectants were selected in the presence of 250 μ g/ml hygromycin (Sigma) and 1–10 μ M methotrexate (Sigma). In the case of LPHFc, stable transfectants were selected in the presence of 1 mg/ml G418 (Invitrogen).

Purification of Recombinant Protein—CHO cells expressing KLFc were cultured in minimum essential medium- α containing 1% ultra low IgG fetal bovine serum (Invitrogen), 100 units/ml penicillin, 0.0001% (w/v) streptomycin, and L-glutamine. Conditioned media were collected after 3 days of culture and centrifuged at 15,000 $\times g$ for 60 min at 4 $^{\circ}C$ to remove debris. Supernatants were filtrated using viva flow 5 and viva spin 20 (pore size; 100 kDa; Viva Science). To measure concentrations of secreted protein (KLFc or LPHFc), a serial dilution of the concentrated media was put into 96-well plates (Falcon) coated with goat anti-human IgG (Fc) antibody (American Qualex) and then incubated with goat anti-human IgG conjugated with horseradish peroxidase (Biosource). After the addition of *o*-phenylenediamine dihydrochloride (Sigma), absorbance at 450 nm was measured by a microplate reader (Bio-Rad). Concentrated conditioned media were precleared twice with Sepharose beads (Amersham Biosciences) and transferred to a new tube with protein A-Sepharose beads (Amersham Biosciences). After an incubation period of at least 2 h at 4 $^{\circ}C$, beads were washed five times with 1 ml of 0.1 M sodium citrate buffer, pH 5.5, containing 0.2 M NaCl, 0.01% Tween 20, and complete protease inhibitor mix (Roche Applied Science) and twice with 1 ml of 0.1 M sodium citrate buffer. The beads bound with KLFc or LPHFc were used for enzymatic studies. Simultaneously, fusion protein quality was checked by SDS-PAGE and SYPRO Orange (Bio-Rad) staining.

Western Blot Analysis—The samples were boiled in SDS-PAGE sample buffer, separated using a 4–20% gradient SDS-polyacrylamide gel (PAG gel; Daiichi Kagaku), and electroblotted onto polyvinylidene difluoride membranes (Immobilon; Millipore). The blot was incubated with KM2119 rat anti-Klotho monoclonal antibody (28) followed by goat anti-rat IgG antibody conjugated with horseradish peroxidase (Biosource). Bands were visualized by exposure to x-ray film (HyperFilm MP; Amersham Biosciences) after incubation with ECL Plus (Amersham Biosciences).

Gel Staining—After SDS-PAGE, gels were incubated at 7.5% acetic acid containing 0.05% SYPRO Orange for 16 h at room temperature. Gels were washed with 7.5% acetic acid for 30 s and double distilled H₂O. Fluorescence intensities were measured by STORM (Molecular Dynamics) using a blue filter.

Detection of Enzyme Activities—We examined the potential substrates for the general β -glucosidases. The standard reaction buffer contained 0.5 mM 4-methylumbelliferyl (4Mu) β -D-glycosides (glucuronide, glucoside, galactoside, *N*-acetylglucosamine, *N*-acetylgalactosamine, fucoside, or mannoside) (Sigma), 0.1 M sodium citrate buffer, pH 5.5, 0.05 M NaCl, 0.01% Tween 20, and 20 μ g of KLFc. The reaction was carried out in a final volume of 100 μ l. Fluorescence intensities were measured at several time points with a multilabel counter ARVOSx (PerkinElmer Life Sciences) at an excitation wavelength of 360 nm and an emission wavelength of 470 nm. Hydrolyzed products were quantified on the basis of 4-methylumbelliferone fluorescence (29). Concentrated conditioned media from nontransfected CHO were used as a negative control after the addition of comparable amounts of purified human IgG1 (Athens Research and Technology). β -Glucosidase from sweet almonds (Sigma), β -glucuronidase from bovine liver (Sigma), and LPHFc were used for reference. To determine optimum pH, a series of 0.1 M sodium citrate buffers, between pH 4.5 and 6.0, and 0.1 M sodium phosphate buffers, between pH 6.0 and 8.0, were used. 0.1–1 mM divalent metal ions were tested by adding CaCl₂, MgCl₂, MnCl₂, CoCl₂, or ZnCl₂ to the standard reaction condition to examine the effects of divalent metal ions. Then we tested other fluorescent substrate conjugated β -glucuronides to confirm β -glucuronidase activity of KLFc. 0.5 mM fluorescein di- β -glucuronide (FD-GlcU) (Molecular Probes) and ELF97TM- β -glucuronide (ELF97-GlcU) (Molecular Probes) were reacted under standard conditions and fluorescence intensities were measured with the same methods as mentioned above. The sensitivity against inhibitors of β -glucuronidase was assayed using 0.1 μ M–1 mM D-saccharic acid 1,4-lactone (Sigma) and taurocholic acid (Sigma) added to the standard reaction condition.

Calculation of Kinetic Constants—Assays to determine the K_m value and V_{max} were performed at 37 °C in reaction buffer containing 0.1 M sodium citrate buffer, pH 5.5, 0.05 M NaCl, 0.01% Tween 20, and 20 μ g of KLFc protein. For determination, various 4Mu-GlcU concentrations between 0.0125 and 3.2 mM were tested. At each incubation time point, 1 μ l of reaction buffer containing reaction products was collected and stopped by the addition of 100 μ l of 0.25 M Na₂CO₃ buffer. Fluorescence intensities were measured as above.

Screening of Natural Substrates—For the screening of natural substrates, steroid glucuronides (β -estradiol 3- β -glucuronide (estradiol-3GlcU), β -estradiol 17- β -glucuronide (estradiol-17GlcU), androsterone glucuronide (androsterone-GlcU), etiocholan-3 α -ol-17-one glucuronide (etiocholan-3 α -ol-17-one-GlcU), testosterone β -glucuronide (testosterone-GlcU), estrone 3- β -glucuronide (estrone-3GlcU), 5 β -pregnane-3 α ,20 α -diol glucuronide (5 β -pregnane-3 α ,20 α -diol-GlcU), dehydroisoandrosterone 3-glucuronide (dehydroisoandrosterone-3GlcU), 5 α -androstane-3 α ,17 β -diol 17 β -D-glucuronide (5 α -androstane-3 α ,17 β -diol 17GlcU), estriol 17 β - β -glucuronide (estriol-17GlcU), estriol 16 α - β -D-glucuronide (estriol-16GlcU), and estriol 3 β -D-glucuronide (estriol-3GlcU) (Sigma), disaccharides of proteoglycan (Chondrosine, IdoUA-2S-GlcNS-6S, IdoUA-GlcNS-6S, IdoUA-GlcNS, IdoUA-GlcNAc-6S, IdoUA-GalNAc, IdoUA-GalNAc-4S, IdoUA-GalNAc-6S, IdoUA-GalNAc-4S, 6S, IdoUA-GlcNAc, GlcA-GalNAc-4S and GlcA-GalNAc-6S) (Seikagaku Kou-gyou Co. Ltd. and Dextra Laboratories, Ltd.), and intact proteoglycans (chondroitin, chondroitin sulfate, keratan sulfate, and heparan sulfate) (Seikagaku Kou-gyou Co. Ltd.) were tested by competitive inhibition assays. For the inhibition assays, each reagent (5 mM) was added to standard reactions including KLFc and 0.1 mM 4Mu-GlcU. To test the hydrolysis of reagents, each substrate (1 mM) was incubated with KLFc fusion proteins under the standard conditions for 30 h. Estradiol-3GlcU and estrone-3GlcU were dissolved into water; etiocholan-3 α -ol-17-one-GlcU, testosterone- β -D-GlcU, and 5 β -pregnane-3 α ,20 α -diol-GlcU were dissolved into ethanol; 5 α -androstane-3 α ,17 β -diol-17GlcU and estriol-3GlcU were dissolved into dimethyl sulfoxide; estradiol-17GlcU, dehydroisoandrosterone-3GlcU, estriol-17GlcU, and estriol-16GlcU were dissolved into *N,N*-dimethylformamide, respectively. To analyze the hydrolysis of the steroid-GlcUs, samples were applied on a HPLC (Waters Alliance HPLC system 2695EX separation module) system equipped with a Waters dual λ UV-visible detector. Symmetry C18 columns (4.6 \times 150 mm; Waters) were used in reversed phase at a flow rate of 1.0 ml/min at 40 °C. 10- μ l samples were injected on the analytical column. The gradient program of mobile phase 1 was as follows: 0.02% trifluoroacetic acid and 10% acetonitrile in water were isocratically eluted for 0 min, then 0.02% trifluoroacetic acid and 80% acetonitrile for 10 min, and 0.02% trifluoroacetic acid and 10% acetonitrile for 10.1 min. The absorbance at 210 or 260 nm was monitored. Finally, we calculated the catalytic parameters for estrane GlcUs. KLFc-catalyzed hydrolysis reactions of estrane-GlcUs were conducted at various substrate concentrations. Catalytic constants were deter-

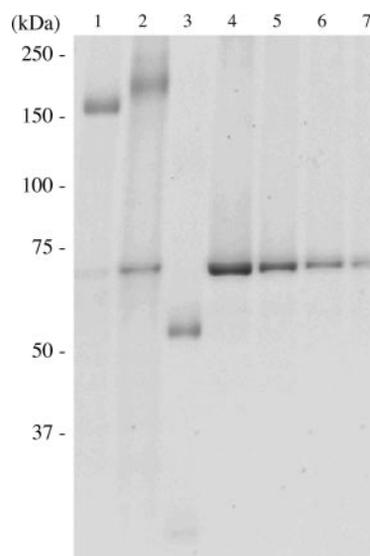


FIG. 2. Assay of the quality and quantity of purified fusion proteins. Conditioned media were collected from KLFc-expressing, LPHFc-expressing, or parental CHO cells. Fc-tagged proteins were purified with protein A beads. Proteins were separated by SDS-PAGE after denaturation under reducing conditions. To visualize whole proteins, the gels were stained subsequently with SYPRO Orange. Lane 1, KLFc; lane 2, LPHFc; lane 3, CHO-conditioned media contained 0.2 μ g of human IgG1 as a control; lanes 4–7, bovine serum albumin used as a standard for estimation of quantity (0.5, 0.25, 0.125, and 0.0625 μ g, respectively).

mined from Lineweaver-Burk plots of three experiments using estrane-GlcUs. To analyze the hydrolysis of chondrosine, amino acid analysis was performed using a Hitachi L-8500 that allows automatic on-line mixing of the ninhydrin reagent. 10 μ l was injected onto the analytical column.

RESULTS

Expression and Purification of Recombinant Klotho—To express recombinant Klotho, we constructed an expression vector using mouse *klotho* cDNA and human immunoglobulin gene. The region encoding the extracellular domain of Klotho was fused to DNA encoding the human immunoglobulin Fc region. For the control experiments, we used mouse LPH, a type 1 membrane protein with two internal repeats homologous to family 1 β -glucosidases. The expression vector for LPHFc was constructed similarly to that for KLFc. These expression constructs were introduced into CHO cells to obtain stable transfectants. Conditioned media from these clones were concentrated and subjected to Western blot analysis. The fusion proteins were detected at relative molecular masses of 150 kDa (KLFc) and 160 kDa (LPHFc), respectively (Fig. 1). Recombinant protein concentrations were estimated by enzyme-linked immunosorbent assay with anti-human IgG antibody. To confirm the reliability of the estimation and to examine the quality of purified fusion proteins, KLFc and LPHFc (200 ng each as estimated by enzyme-linked immunosorbent assay) were separated by SDS-PAGE, and the gel was stained with SYPRO Orange to visualize proteins (Fig. 2). The KLFc protein was detected as a single band, indicating that this fusion protein could be purified without proteolysis (Fig. 2, lane 1). The LPHFc protein could also be purified (lane 2). The weakly detected 70 kDa bands are likely contamination of bovine serum albumin from fetal bovine serum. A bovine serum albumin serial dilution was used as a standard for quantification (Fig. 2, lanes 4–7). Signal intensities were compared with standards to calculate the quantities of KLFc and LPHFc. The results correlated well with those obtained by enzyme-linked immunosorbent assay. We used the purified fusion proteins for the following analysis.

TABLE I
Measurement of the enzymatic activity of recombinant KLFc protein

Enzymatic activity on 4Mu-glycosides is shown. These data represent the results of three independent experiments. Values \pm S.D. were obtained after calculation from the results of a reaction for 3 h. Rat LPHFc, bovine liver β -glucuronidase, and almond β -glucosidase were used as positive controls. Human IgG1 was used as a negative control. Fuc, fucose; Xyl, xylose.

Enzyme	Substrate							
	Glc	Gal	GlcNAc	GalNAc	GlcU	D-Fuc	Man	Xyl
	<i>pmol/h/μg</i>							
Klotho	0.34 \pm 0.13	1.34 \pm 0.42	1.71 \pm 0.96	1.43 \pm 0.27	72.8 \pm 6.79	0.23 \pm 0.11	0.91 \pm 0.72	0.61 \pm 0.27
LPH	1,103.4 \pm 73.6	188.9 \pm 3.98	6.5 \pm 2.55	6.85 \pm 0.9	202.5 \pm 39.48	19,400.4 \pm 923.8	260.9 \pm 60.8	106.4 \pm 21.8
IgG	0.70 \pm 0.03	0.19 \pm 0.18	1.61 \pm 0.12	0.80 \pm 0.21	0.44 \pm 0.14	0.10 \pm 0.06	0.44 \pm 0.38	0.38 \pm 0.17
β -Glucosidase	51,070 \pm 2,892	1,232.8 \pm 85.8	1,293.5 \pm 39.5	489.7 \pm 287.2	91.0 \pm 146.4	45,036 \pm 1,808.7	162.5 \pm 115.6	1,317.3 \pm 724.3
β -Glucuronidase	34 \pm 2.5	491.7 \pm 26.8	1669.3 \pm 66.0	1444.4 \pm 97.6	1940.3 \pm 23.1	38.2 \pm 3.11	17.0 \pm 3.0	11.7 \pm 3.78

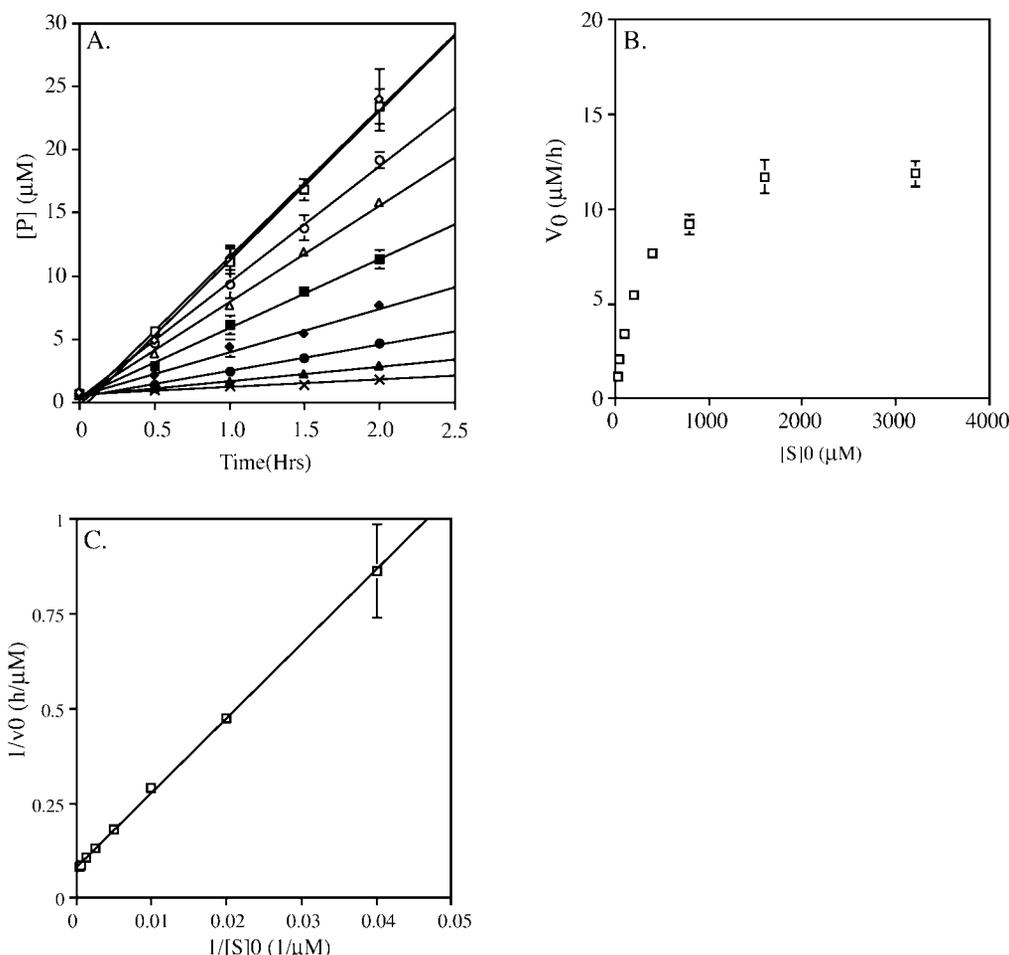


FIG. 3. Calculation of catalytic constants for KLFc as a β -glucuronidase. A, 20 μ g of recombinant KLFc protein was incubated with serial concentrations of 4Mu-GlcU (0.0125–3.2 mM) at 37 $^{\circ}$ C. Open square, 3.2 mM; open rhombus, 1.6 mM; open circle, 0.8 mM; open triangle, 0.4 mM; closed square, 0.2 mM; closed rhombus, 0.1 mM; closed circle, 0.05 mM; closed triangle, 0.025 mM; cross, 0.0125 mM. B, substrate saturation curve for KLFc; initial velocity (v_0) and initial concentration of substrate ($[S]_0$) were plotted. The x and y axes represent initial concentration of substrate (μ M) and initial velocity (μ M/h), respectively. C, Lineweaver-Burk plots for KLFc. Enzymatic activity of KLFc was assayed at pH5.5 using 4Mu-GlcU over a 200-fold concentration range. The x and y axes indicate the reciprocals of the initial concentration of substrate ($1/[S]_0$; μ M $^{-1}$) and initial velocity ($1/v_0$; h \cdot μ M $^{-1}$), respectively. Error bars represent S.D. of three experiments.

Enzymatic Activity of Klotho—To investigate the enzymatic activity of the KLFc protein, a panel of 4Mu-glycosides was tested. These substrates are not fluorescent, but their hydrolysis liberates fluorescent 4-methylumbelliferone, which was measured using a fluorometer as a function of time to estimate the quantity of hydrolyzed substrate. Eight types of glycosides were tested. KLFc reacted specifically with 4Mu-GlcU and hydrolyzed this substrate.² Changes in the fluorescence intensity of other potential substrates were similar to the back-

ground levels observed with IgG1 (Table I), indicating that KLFc does not hydrolyze these molecules. On the other hand, LPHFc, β -glucosidase, and β -glucuronidase were active on a set of substrates as shown in Table I. Table I shows that the specific activity of Klotho is about 30 times less than that of β -glucuronidase from bovine liver and one-third of that from rat LPH. To exclude clonal variation, we tested three other independently established transfectants and confirmed that fusion proteins obtained from these clones gave rise to similar results in a dose-dependent manner.² Furthermore, 0.5 mM FD-GlcU and ELF97-GlcU, the other fluorescent substrate-conjugated β -glucuronic acids, were tested and found to be

² O. Tohyama, A. Imura, A. Iwano, J.-N. Freund, B. Henrissat, T. Fujimori, and Y.-i. Nabeshima, unpublished data.

TABLE II
Catalytic constants of KLFc protein as a β -glucuronidase using 4Mu-GlcU as the substrate

Catalytic constants were determined from linear Lineweaver-Burk plots (Fig. 3C) of three experiments.

	K_m	V_{max}	k_{cat}	k_{cat}/K_m
	mM	mM/h	1/h	1/mMh
4 Mu-GlcU	0.249 ± 0.071	0.0124 ± 0.00173	21.8	87.48

hydrolyzed under the same conditions, thus confirming the β -glucuronidase activity of KLFc proteins.²

Calculation of Rate Parameters—Substrate saturation for the KLFc fusion protein was examined with a series of 4Mu-GlcU concentrations. The concentration of products ($[P]$) and reaction time (t) were plotted as shown in Fig. 3A. 3.2 and 1.6 mM substrate resulted in a similar level of products, suggesting that substrate saturation was reached at 1.6 mM for 20 μ g of KLFc protein. Based on the slope of these lines, the initial velocity (v_0) was calculated for each initial concentration of substrate ($[S]_0$). $[S]_0$ and v_0 are plotted in Fig. 3B. The reciprocal Lineweaver-Burk plot is shown in Fig. 3C. The Michaelis constant (K_m) and maximum velocity (V_{max}) were calculated from this plot and are shown in Table II. Fig. 3B and Table II show that the reaction of KLFc fusion protein on 4Mu-GlcU fits Michaelis-Menten kinetics.

Optimal pH and Effects of Divalent Metal Ions—We examined the effects of pH on the enzymatic activity of KLFc (Fig. 4). Sodium citrate buffer was used between pH 4.5 and 6.0, and sodium phosphate buffer was used between pH 6.0 and 8.0 to determine the optimum conditions between pH 4.5 to 8.0 by 0.5 increments. The highest activity of KLFc for the processing of 4Mu-GlcU was seen at pH 5.5, suggesting that this is the optimal pH for KLFc. Because dependence on metal ions for the activity of some glucosidases has been reported (30, 31), we examined the effects of Ca^{2+} , Mg^{2+} , Mn^{2+} , and Zn^{2+} on the enzymatic activity of Klotho. However, none of these metal ions appeared to have an effect on the enzymatic activity of KLFc.²

Sensitivity to Inhibitors—To characterize further the KLFc fusion protein, we examined the effects of known inhibitors for β -glucuronidase on its enzymatic activity (Fig. 5). Bovine liver β -glucuronidase was used as a standard to measure the differences between known enzymes. D-Saccharic acid 1,4-lactone and taurocholic acid, which are known competitive inhibitors for bovine liver β -glucuronidase, were added to 4Mu-GlcU hydrolysis reaction solutions. As shown in Fig. 5, A and B, KLFc enzymatic activity was reduced by these inhibitors. These inhibitory effects were also observed on β -glucuronidase, but at a lower dose than was required for KLFc. These results indicated that the enzymatic activities of KLFc were inhibited with known inhibitors of β -glucuronidase.

Candidates for Klotho Substrates—To search for Klotho potential native substrates, we screened natural glucuronide conjugates including proteoglycans (32), steroid hormones (33, 34), glycolipids (35), alkaloids (36), and flavonoids (37). We first examined whether these compounds could competitively inhibit the hydrolysis action by the KLFc fusion protein. Each compound (5 mM) was added to reactions containing 10 μ g KLFc and 0.1 mM 4Mu-GlcU. Disaccharides derived from partial lysis of proteoglycan or intact proteoglycans (chondroitin, chondroitin sulfate, keratan sulfate, and heparan sulfate) had no effect. Among 11 steroid-GlcUs tested, eight (androsterone-GlcU, etiocholan-3 α -ol-17-one GlcU, estrone-3GlcU, estradiol-3GlcU, 5 β -pregnane-3 α ,20 α -diol-GlcU, dehydroisoandrosterone-3GlcU, testosterone-GlcU, and estriol-3GlcU) showed inhibitory effects on the hydrolysis of 4Mu-GlcU by KLFc (Fig. 6). No proteoglycan inhibited the hydrolysis of 4Mu-GlcU.² We

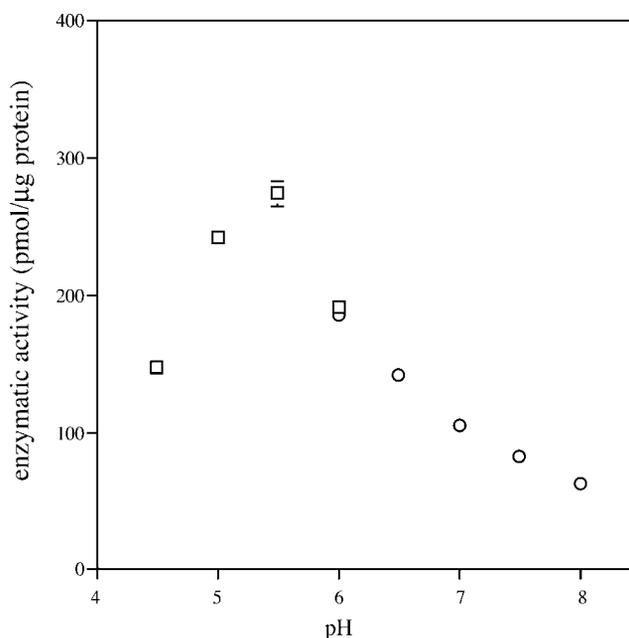


FIG. 4. Effect of pH on the β -glucuronidase activity of KLFc. β -Glucuronidase activity of KLFc was assayed using 4Mu-GlcU at various pH conditions from 4.5 to 8.0. The reaction was carried out at 37 °C for 6 h. 0.1 M sodium citrate buffer (square) was used at pH between 4.5 and 6.0. 0.1 M sodium phosphate buffer (circle) was used between 6.0 and 8.0. The highest activity was observed at pH 5.5. Error bars represent S.D. of three experiments.

further tested whether steroid-GlcUs (estradiol-3GlcU, estrone-3GlcU, estriol-3GlcU, testosterone-GlcU, and androsterone-GlcU) could be hydrolyzed with KLFc. Each glucuronide was incubated with KLFc fusion protein. The reaction products were applied on a HPLC gradient chromatogram and detected with absorbance at 210 or 260 nm. As a result, KLFc hydrolyzed estradiol-3GlcU, estrone-3GlcU, and estriol-3GlcU, with catalytic parameters (k_{cat} and k_{cat}/K_m) similar to those obtained for 4Mu-GlcU (Tables II and III). Testosterone-GlcU was barely hydrolyzed by KLFc, whereas androsterone-GlcU and chondrosine were not hydrolyzed at all.²

DISCUSSION

In the present study, recombinant Klotho was found to exhibit a subtle but specific activity as a β -glucuronidase. We had difficulty in purifying sufficient amounts of native Klotho required for our enzyme studies because of its low expression levels *in vivo* and its increased instability during preparation. We therefore employed a unique system of protein preparation using CHO cells and the construction of a chimeric protein fused to Fc region of the human IgG1 gene. This method produced a secreted Klotho protein that was soluble and stable in various solutions and could be purified easily using protein A beads. As a result, significant β -glucuronidase activity could be detected in our assay systems using 4Mu-GlcU. Other fluorescent glucuronides (FD-GlcU and ELF97-GlcU) were also hydrolyzed with KLFc. Inhibitors of β -glucuronidase, D-saccharic acid 1,4-lactone and taurocholic acid, were also found to reduce the enzymatic activity of Klotho. Taken together, recombinant Klotho can be categorized as a β -glucuronidase by its substrate specificity and sensitivity against inhibitors. Interestingly, the CAZy data base, which classifies glycoside hydrolases in families based on amino acid similarity, places Klotho in glycosidase family 1. This family groups together different enzymes, including β -glucosidase (EC 3.2.1.21), β -galactosidase (EC 3.2.1.23), β -mannosidase (EC 3.2.1.25), and β -D-fucosidase (EC 3.2.1.38), but to date, no β -glucuronidase members have been

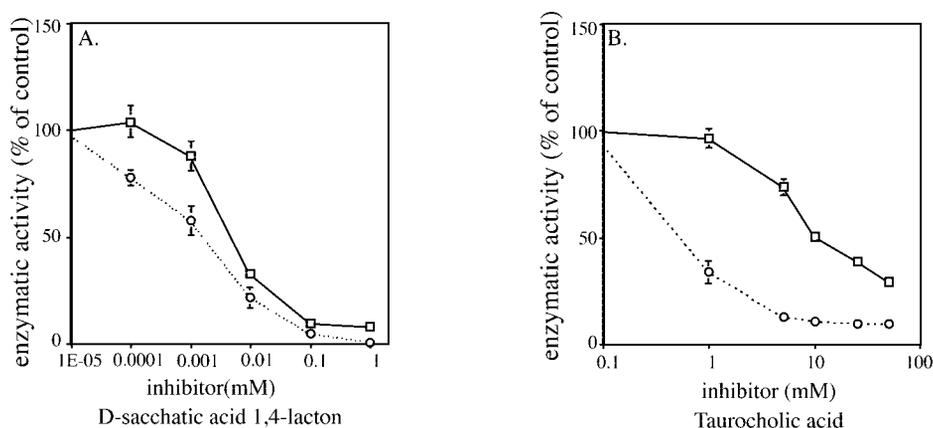


FIG. 5. **Sensitivity of KLFc against inhibitors.** 20 μ g of KLFc or control β -glucuronidase was incubated with 4Mu-GlcU in presence of various concentrations of β -glucuronidase inhibitors. A, D-saccharic acid 1,4-lactone; B, taurocholic acid. Square, KLFc; circle, β -glucuronidase. Relative enzymatic activity of each protein is shown based on the activity without addition of inhibitor. Error bars represent S.D. of three experiments.

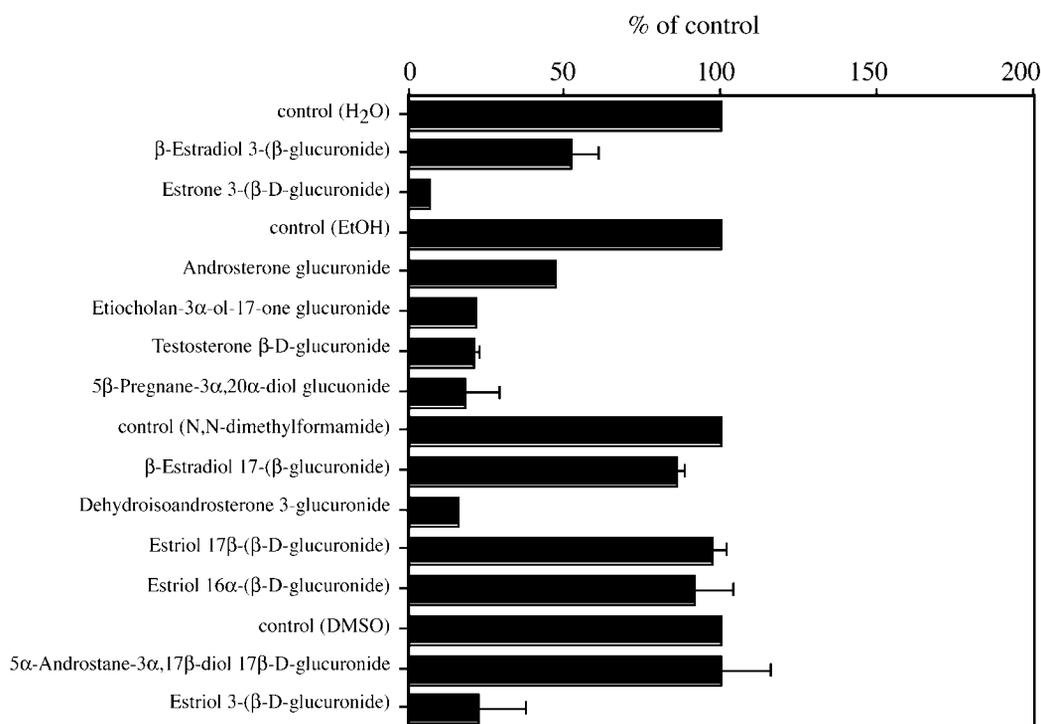


FIG. 6. **Inhibition assay of natural glucuronides.** Each of the steroid glucuronides, disaccharides of proteoglycan, and intact proteoglycans, described under "Experimental Procedures," was added separately to the reaction of KLFc (10 μ g) and 4Mu-GlcU (0.1 mM). The reaction was carried out at 37 $^{\circ}$ C for 6 h. The reaction with added solvent (H_2O , ethanol, dimethyl sulfoxide (DMSO), and *N,N*-dimethylformamide) was used as control and referred to as 1. Error bars represent S.D. of three experiments.

TABLE III

Catalytic constants of KLFc protein using estrane-GlcUs as substrates

Hydrolyses of estrane-GlcUs with KLFc protein were conducted at various substrate concentrations. Catalytic constants were determined from linear Lineweaver-Burk of three experiments.

Substrate	K_m mM	k_{cat} 1/h	k_{cat}/K_m 1/h/mM
Estrone 3-(β -D-glucuronide)	0.251 \pm 0.021	29.24	116.7
β -Estradiol 3-(β -D-glucuronide)	0.174 \pm 0.030	35.01	201.8
Estriol 3-(β -D-glucuronide)	0.251 \pm 0.008	32.80	130.9

reported. Klotho therefore represents the first enzyme with β -glucuronidase activity in this family. Further studies, such as three-dimensional structure determination, will be necessary to understand the basis of this unusual specificity because all previously known β -glucuronidases belong to the glycosidase family 2 (3–5). Phylogenetic analysis based on the neighbor-joining method (38) using ClustalW software suggests dis-

tant similarity between Klotho and β -glucuronidase.² However, the discovery of a novel substrate specificity such as β -glucuronidase in family 1 glycosidases is not surprising given the resemblance between the various substrates in this family (they are all β -glycopyranosides), and the occurrence of glycosidases of differing substrate specificity in the same family illustrates a common evolutionary origin (3–5, 39). In consequence, and as noted previously, the glycosidase families based on sequence (hence three-dimensional and mechanistic) similarity does not coincide with the earlier classification of glycosidases based on their substrate specificity (3–5, 39). Furthermore, recent studies on sequence comparisons together with three-dimensional structure determination and mechanism elucidation have shown that several glycosidase families could be grouped together in a large superfamily called "clan GH-A," which includes families 1, 2, 5, 10, 17, 26, 30, 35, 39, 42, 51, 53, 59, 72, 79, and 86 (39, CAZY data base). Altogether this clan groups a wide range of substrate specificities including β -glu-

uronidases. In addition, novel members structurally related to glycosidases, but not isofunctional to their glycosidase ancestors, have recently emerged (40). A vivid example is soybean hydroxyisourate hydrolase (41), which shows extensive sequence similarity to glycosidase family 1, including conservation of the known catalytic residues. Interestingly, the catalytic residues of this enzyme are conserved and have kept their original roles, but they catalyze a different reaction on a non-carbohydrate substrate (41).

How Klotho performs its catalytic action despite an apparently crippled catalytic machinery is not clear. Several scenarios can be envisioned (including substrate-assisted catalysis, change from retaining to inverting mechanism, presence of a cofactor that would replace a catalytic residue, domain swapping), but all are speculative in the absence of a detailed mechanistic and/or three-dimensional characterization. A further layer of complexity is generated by the two-domain structure of Klotho, in which half of the catalytic machinery is missing in the first domain, and the other half is missing in the second domain. In an attempt to address these questions, we tried to examine the effects of mutagenesis of the "remaining" catalytic residue in each repeat of Klotho to identify which of the two repeats contains the activity. However, the mutated proteins were stacked in the cells, and it was impossible to analyze the enzymatic properties of the Klotho mutants. We also tested the effect of L-ascorbic acid, the cofactor of myrosinase, but no effect was detected on the enzymatic activity of Klotho.² However, the possibility of a yet unknown other cofactor for Klotho cannot be excluded.

The enzymatic activity of Klotho showed maximal activity at pH 5.5, a value close to the pH 6.0 optimal for bovine liver β -glucuronidase. However, although the enzymatic activity of β -glucuronidase was inhibited by citrate buffer, that of Klotho was unaffected. In addition, the enzymatic activity of Klotho was reduced by known inhibitors of β -glucuronidases. Based on these data, we conclude that Klotho is a β -glucuronidase.

We then screened for natural β -glucuronides that may be hydrolyzed by Klotho. We found that some steroid β -glucuronides could competitively inhibit the β -glucuronidase activity of Klotho. Furthermore, we observed that Klotho can hydrolyze certain steroid β -glucuronides such as estradiol-3GlcU, estrone-3GlcU, and estriol-3GlcU. Both 4Mu-GlcU and estrane-GlcUs share a common phenolic aglycone structure, with the sugar attached to the C-3 of the A ring in these cases, suggesting that Klotho is specific for the aglycone. Although further investigation is necessary to identify the natural substrate of Klotho, β -glucuronides with a phenolic aglycone are good candidates, particularly steroid-GlcUs. Many undefined but biologically active steroids are predicted to exist in the animal body. As steroid hormones, some of these substances potentially undergo β -glucuronide conjugation. These steroid-GlcUs would be suitable candidate substrates for Klotho, which could process them into bioactive steroids possessing multipotent functions required for the regulation of many biological phenomena related to *kl*^{-/-} phenotypes. Furthermore, humoral factors such as steroids might be appropriate and favorable for the systemic and synchronized actions of Klotho which are predicted from its biological roles and the phenotypes of *kl*^{-/-} mice.

The kinetic parameters of Klotho were calculated using 4Mu-GlcU. The K_m value of Klotho (0.249 mM) is higher than that of β -glucuronidases purified from human placenta (0.045 mM) and serum (0.06 mM), respectively (42), suggesting that the affinity of Klotho for this artificial substrate is lower than that of known β -glucuronidases. In addition, we calculated the kinetic constants (k_{cat} and k_{cat}/K_m) of natural substrates (estradiol-

3GlcU, estrone-3GlcU, and estriol-3GlcU) and compared them with those of 4Mu-GlcU. The kinetic constants of estradiol-3GlcU, estrone-3GlcU, and estriol-3GlcU were of the same order of magnitude as those of 4Mu-GlcU. These results suggest that Klotho has an enzymatic activity considerably lower than that of other glycosidases. It is possible that the observed activity of Klotho is adequate for its *in vivo* biological function(s). However, we cannot exclude the possibility that Klotho might act more efficiently in combination with prosthetic groups such as cofactors and/or coenzymes. Further studies including the identification of authentic substrates are necessary for the definitive discussion of the activity of Klotho. We also examined the binding ability of Klotho to various substrates using tritiated β -glucuronic acid to test the possibility that Klotho may function as a sugar-binding protein. However, no signals could be detected after gentle washes, indicating that the predominant role of Klotho is likely to be an enzyme rather than a binding protein.²

Glucuronylation generally increases the polarity, the water solubility, and the excretability of toxic biological substances. Their hydrolysis by Klotho is therefore a reversal of glucuronidation. It is tempting to speculate that Klotho may convert inactive glucuronylated steroids to active forms that are critical for the maintenance of calcium homeostasis and protection of tissues from various impairments observed in *klotho* null mice. Although the native substrate of Klotho is yet to be established, it is likely that the action of Klotho could be mimicked by the addition of the deconjugated form of the putative steroid-GlcU(s).

Despite numerous analyses of the aging-related phenotypes of *klotho* null mice over the recent years, the molecular mechanisms underlying Klotho function remain unclear. This study is the first to show that Klotho can act as an enzyme *in vitro* and provides helpful information in the search for natural substrate(s) and in delineating physiological and pathological roles of Klotho proteins in maintenance of a healthy state and in aging-associated disorders.

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