Secreted Klotho protein in sera and CSF: implication for post-translational cleavage in release of Klotho protein from cell membrane

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Abstract ***Klotho*** **mutant mice exhibit a set of phenotypes resembling human ageing. Although the function of Klotho remains unclear, mediation of its pleiotropic functions by putative humoral factor(s) has been presumed. Newly established antibodies against Klotho allowed the detection of secreted Klotho, a candidate for the putative humoral factor, in sera and cerebrospinal fluid. Surprisingly the secreted Klotho was 130 kDa, in contrast to the 70 kDa predicted form from** ***klotho*** **gene transcripts. The secreted as well as the membrane-bound Klotho proteins were suggested to form oligomerized complex. These results delineate post-translation processing of Klotho and possible regulatory mechanisms for secretion of Klotho in vivo.**

Keywords: Klotho protein; Humoral factor; N-glycosylation; Post-translational cleavage

1. Introduction

klotho mutant mice were originally described as an animal model harboring a set of phenotypes resembling human ageing [1]. The *klotho* (*kl*) gene encodes a type I membrane protein with an extracellular domain possessing two internal repeats (KL1, KL2) that share homology to family 1 β-glycosidase [2]. In *kl/kl* mice, some of the major changes are thought to occur in a cell-non-autonomous fashion, because *klotho* is predominantly expressed in the kidney, the parathyroid gland, and the choroid plexus, but not detectable in many organs that are severely affected. In addition, transgenic expression of *klotho*, though limited to brain and testis, improved macroscopic abnormalities observed in *kl/kl* mice [1]. The limited delivery of Klotho and the exchange of humoral elements by parabiosis showed some protective effects in vivo [3–5]. Taken together, these findings suggested that functional deficiency of extracellular factor(s) should be involved in the pathogenesis shown in *klotho* mutant mice.

An alternatively spliced form of *klotho* mRNA, encoding the N-terminal half of Klotho (KL1), was detected in humans [6] and mice [7] and led to the hypothesis that a secreted form of Klotho existed extracellularly. It was also speculated that this putative secreted KL1 protein might play a significant role as a humoral factor to protect organs from various impairments.

In the present study, we developed new monoclonal antibodies (mAbs) against Klotho and succeeded in detecting a 130 kDa Klotho protein in both serum and cerebrospinal fluid (CSF). Secreted KL1 predicted from *klotho* gene transcripts was not detected in extracellular fluid. Here, we report the molecular characterization of Klotho proteins.

2. Materials and methods

2.1. Plasmid construction

The expression vectors for full-length mouse Klotho fused with EGFP, hemagglutinin (HA) or Flag tag at the C-termini were constructed. In order to introduce a translational initiation sequence and an *Eco*RI site to the 5′ region of mouse *klotho* cDNA, the 5′ end of *klotho* cDNA, termed mKL-N, was amplified via PCR using a set of primers; mKL-S1, 5-aaagaattcgccaccatgctagcccgcgcc-3 (*Eco*RI and Kozak initiation sequences are underlined) and mKL-AS1, 5-catggctgaattctcccagtcag-3. To delete the stop codon and add a *Bam*HI site into the 3′ region, a set of primers; mKL-S3, 5-aggaggatccgatgaaatacaacg-3 (*Bam*HI site is underlined) and mKL-AS3, 5-tttctcgagcttataacttctctggcctttcttgg-3 (*Xho*I site is underlined) were used to amplify the 3′ end of *klotho* cDNA, termed mKL-C. The *Eco*RI–*Bam*HI fragment of mKL-N and *Bam*HI–*Xho*I fragments was ligated and cloned into *Eco*RI–*Xho*I sites of pSP72 giving PSP72-*klotho*. Then, the *Eco*RI–*Xho*I fragment of PSP72-*klotho* was excised and inserted into pEGFP-N2 (Clontech), pcDNA3-HAC and pcDNA3-FlagC (Invitrogen) to give KL-EGFPC, KL-HAC and KL-FlagC, respectively.

2.2. Animals

klotho null mice are described elsewhere [8]. C57BL/6 mice and Sprague–Dawley rats were purchased from CLEA Inc. Japan. All the animals were maintained in SPF conditions according to the institute's guidelines.

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Abbreviations: SDS–PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; CSF, cerebrospinal fluid; HA, hemagglutinin; FBS, fetal bovine serum; HRP, horseradish peroxydase; CHO, Chinese hamster ovary; MEM, minimum essential medium; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; RT, room temperature; mAb, monoclonal antibody

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2.3. Cells and primary organ culture conditions

Chinese hamster ovary (CHO) cells were cultured in minimum essential (MEM) alpha medium (Sigma) containing 1% (v/v) fetal bovine serum (FBS), 100 U/ml of penicillin, 0.0001% (w/v) streptomycin and 2 mM L-glutamine (Sigma). Four micrograms of plasmid was introduced into 10^7 cells using LipofectAMINE PLUS reagent (Invitrogen) and geneticine (Invitrogen) resistant colonies were selected. CHOTM2, a high producer of full-length Klotho, was a gift of KYOWA Hakko Kogyo, Co. Ltd. Mouse kidneys and choroid plexus were dissected, suspended in phosphate-buffered saline (PBS), and cultured in RE-BM™ medium (Clonetics) for 24 h at 37 °C in a CO_2 incubator. Choroid plexus were treated with 0.5 mg/ml of EZ-Link™ Sulfo-NHS-LC-Biotin (Pierce) for 30 min on ice to biotinylate cell surface proteins, rinsed twice with cold PBS and then cultured.

2.4. Monoclonal antibodies

All mAbs were developed as described elsewhere [9]. Hybridoma supernatants were screened based on histological staining affinities for *klotho*-transfected CHO cells and abilities for immunoprecipitation (IP) of recombinant Klotho proteins. 131 mAb is a mouse IgG1 antibody against human OX40 [9] and used as a subclass matched control. Purified mAbs were conjugated to hydrazide beads (Bio-Rad) according to the manufacturer's instructions. For IPs of HA-tagged and Flag-tagged Klotho proteins, anti-HA agarose conjugated beads (Sigma) and anti-FLAG M2 affinity gel (Sigma) were purchased, respectively.

2.5. Western blot analysis

Kidneys were homogenized and lysed in lysis buffer containing 20 mM HEPES, pH 7.4, 150 mM NaCl, Complete™ (Roche) and 2% CHAPS and incubated at 4 °C overnight. Ten micrograms of supernatant proteins (14 000 rpm for 30 min) measured by DC protein assay kit (Bio-Rad) was analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) and electrically transferred onto PVDF membranes (Immobilon; Millipore). The blot was incubated with 1 μg/ml of monoclonal anti-Klotho antibodies for 60 min and then incubated with horseradish peroxidase (HRP)-conjugated goat anti-rat Ig (1:5000, Bio Source) for 30 min. Bands were visualized using the ECL plus system (Amersham Biosciences). Choroid plexus were treated with the above lysis buffer containing 4% CHAPS overnight to extract soluble proteins. About 2 μg proteins from the supernatants were analyzed by Western blotting.

2.6. Immunoprecipitation from serum or CSF

Blood samples were obtained from human volunteers and mice. Two human CSF samples from therapeutic drainage performed at Kyoto University Hospital were utilized for this experiment according to the institute's guidelines. Serum and CSF were diluted with an equal volume of buffer containing 20 mM Tris–HCl, pH 7.5, 150 mM NaCl, 0.5% Triton X-100 and 2 mM ethylenediaminetetraacetic acid (EDTA) and filtered with 0.45 μm millex filter (Millipore). One milliliter of diluted serum and CSF samples were incubated with 20 μl of beads conjugated with Mink1 (mouse antibody) or 131 mAb, a control antibody at 4 °C for 3 h. Incubated beads were washed five times with a buffer containing 20 mM Tris–HCl, pH 7.5, 200 mM NaCl, 0.5% Triton X-100 and 2 mM EDTA. Immunoprecipitated proteins were eluted by incubation at 95 °C for 5 min in 20 μl of 2× sample buffer (60% (v/v) glycerol, 4% SDS, 10 mM EDTA and 125 mM Tris, pH 6.8) without a reducing reagent and 10 mM dithiothreitol (DTT) was added after removal from the beads.

2.7. Immunoprecipitation from transfectants

Cos-7 cells transiently transfected with expression vectors for KL-HAC, KL-FlagC, or both were washed twice with cold PBS and lysed in ice-cold cell lysis buffer (20 mM HEPES, pH 7.4, 1% Triton X-100 and 150 mM NaCl containing a Complete™). Supernatants of lysates at 14 000 rpm for 30 min were incubated with Sepharose 4B (Amersham Bioscience) for 1 h to remove non-specifically bound materials. For IPs, 10 μl of agarose beads conjugated with monoclonal anti-HA antibody (Sigma) or anti-FLAG M2 affinity gel (Sigma) was incubated with 100 μl of pre-cleared lysate for 16 h. The precipitants were then washed with buffer containing 20 mM HEPES, pH 7.4, 150 mM NaCl and 0.5% Triton X-100. Proteins were eluted after incubation with 2× sample buffer at 95 °C, applied to SDS–PAGE and then transferred to Immobilon-P (Millipore). The immunoblots were probed with the anti-HA or anti-Flag M2 antibody and then developed with HRP-conjugated donkey anti-mouse IgG (Amersham Bioscience) or HRP-conjugated sheep anti-mouse IgG (Amersham Bioscience) and exposed to hyperfilm™ (Amersham Bioscience) using chemiluminescence ECL plus reagent.

2.8. Size exclusive chromatography

Twenty milliliters of culture supernatant of CHOTM2 was passed through 0.22 μm filter and concentrated to 0.5 ml with size exclusive spin columns (Vivascience). The samples (50 μl) were applied to a TSK-3000SW_{XL} column (TOSOH, Japan) equilibrated with 100 mM sodium phosphate buffer (pH 6.8) containing 200 mM NaCl and 2 mM EDTA using a liquid chromatography system (BioCAD 700E, Applied Biosystems) at a flow rate of 0.5 ml/min at room temperature (RT). Fractions (100 μl) were collected and analyzed by Western blot with KM2076 using a chemiluminescence imaging analyzer (LAS3000mini, FUJI Film, Japan). The molecular weights of eluted Klotho proteins were estimated with molecular weight standards (Gel Filtration Standard, Bio-Rad) monitored by UV absorbance at 280 nm.

3. Results

3.1. Establishment of mAbs

We established a series of mAbs recognizing Klotho proteins in order to analyze whether Klotho exists in extracellular fluid. Newly established (Rink6, Rink12, Rink14, Rink107 and Mink1) and previously established (KM2076 and KM2119) mAbs [10] were characterized (Table 1). KM2076 and KM2119 are both useful for Western blot analysis to detect 120, 130 and 135 kDa Klotho proteins. On the contrary, IP of lysates from mouse kidney and choroid plexus with KM2076 and KM2119 gave a single band of 120 kDa. Newly established mAbs were all useful for immunohistochemistry (IHC) and IP of Klotho proteins, but not for Western blotting.

Table 1
Characterization of mAbs

MAb	Animal	Subclass	Antigen	Recognition site	Western blot	IHC	IP
KM2076	Rat	IgG2a	N-KL-GST	KL1	Yes	+	+[a]
KM2119	Rat	IgG2b	C-KL-GST	KL2	Yes	+	+[a]
Mink1	Mouse	IgG1	rKL	KL1	No	++	+++
Rink6	Rat	IgG2b	rKL	KL1	No	++	+++
Rink12	Rat	IgG2a	rKL	KL1	No	+++	+++
Rink14	Rat	IgG2b	rKL	KL1	No	+++	++
Rink107	Rat	IgG2a	rKL	KL1	No	++	+++

[a] KM2076 and KM2119 give a single band with 120 kDa in IP method. +, ++ and +++ represent intensities of fluorescence signals for IHC and efficiency of IP. N- or C-KL-GST: N-terminal or C-terminal recombinant Klotho proteins fused with GST protein from *E. coli* lysates, respectively. rKL: recombinant mouse Klotho protein from CHO supernatants.

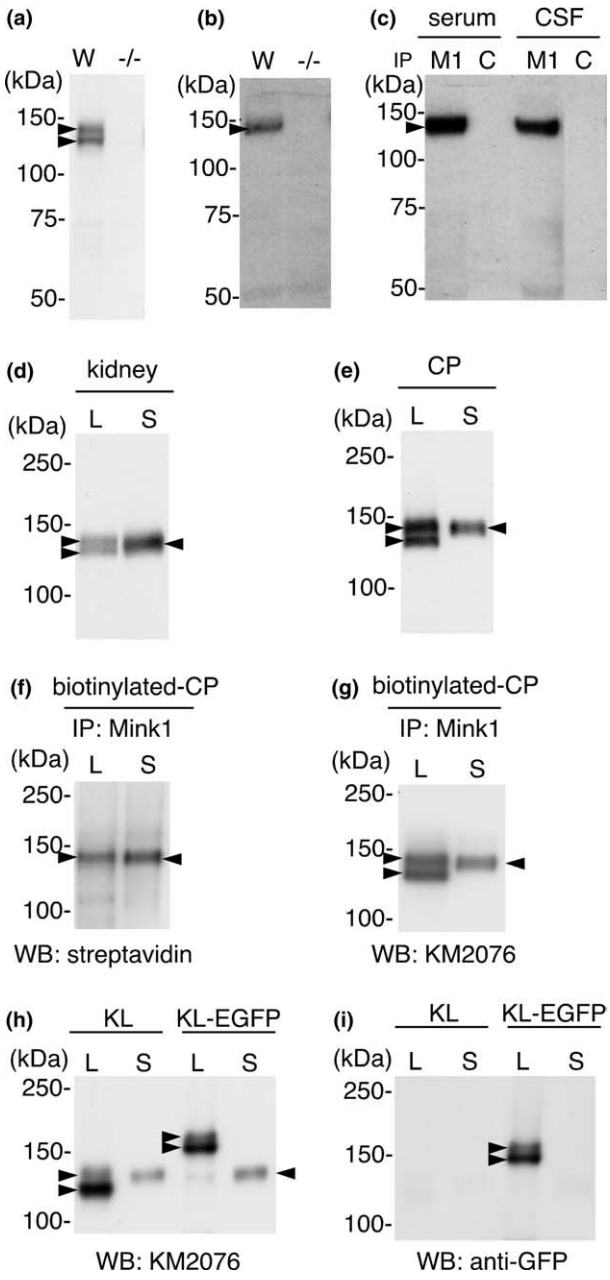

Fig. 1. Characterization of secreted Klotho. (a) Lysates of kidneys from female C57BL/6 (W) and *kl*–/– mice were subjected to Western blotting with KM2076 following SDS–PAGE with 7.5–15% gradient polyacrylamide gel. (b) Klotho protein in mouse sera was immunoprecipitated using Mink1-conjugated beads and (c) human sera and CSF were used for immunoprecipitation with Mink1 (M1) or with control antibody (C), and subjected to Western blot analysis with KM2076. (d, e) Klotho in lysates (L) and organ culture supernatants (S) of kidneys and choroid plexus (CP) from C57BL/6 mice were immunodetected with KM2076 as described in (a). In both lysates, two bands corresponding 120 and 135 kDa were detected (arrowheads). (f, g) Choroid plexus were labeled with biotin for 30 min in PBS on ice. Cell lysate (L) was prepared just after labeling. In addition, labeled choroid plexus were cultured for 3 h and the conditioned media were used for immunoprecipitation with Mink1 anti-Klotho antibody (S). These samples were immunoblotted with either HRP-conjugated streptavidin (f) or KM2076 mAb (g). (h, i) Mouse *klotho* and *klotho*-EGFP expression plasmids were transfected into CHO cells, respectively, and the lysates and the supernatants were immunodetected with either KM2076 mAb (h) or anti-GFP antisera (i).

3.2. Detection of secreted Klotho protein

To characterize Klotho proteins, lysates of kidneys and immunoprecipitants of sera from C57BL/6 and *kl*–/– mice were examined by Western blot analysis using KM2076 mAb. Distinct bands were seen in the kidney lysate (120 and 135 kDa) and immunoprecipitants (130 kDa) from sera of C57BL/6 mice but not in those from *kl*–/– mice (Fig. 1(a) and (b)). The same method was applied for the analysis of human sera and CSF. A unique band of 130 kDa was detected in the IP with Mink1-conjugated beads, but not with negative control beads (Fig. 1(c)), demonstrating that a soluble Klotho protein of 130 kDa exists in extracellular fluids in humans and mice.

3.3. Release of Klotho protein by proteolysis

To assess the possibility of release of membrane-bound Klotho protein, we examined lysates and supernatants using mouse kidney and choroid plexus. In both organs, dual bands of 120 and 135 kDa were detected from lysates, while a single band of 130 kDa was seen in supernatants (Fig. 1(d) and (e)). To further characterize the secretion of Klotho protein, cell surface molecules on mouse choroid plexus cells were labeled with water soluble-biotin, immunoprecipitated with Mink1-conjugated beads, and applied to Western blot analysis. The upper 135 kDa band was obviously biotinylated. On the contrary, the lower band (120 kDa) was not detected with streptavidin (Fig. 1(f)), but detectable when the same membrane was visualized with KM2076 (Fig. 1(g)). These results suggest that the biotinylated upper band represents the cell surface form of Klotho and the lower 120 kDa band should be the intracellular form of Klotho. 130 kDa of Klotho protein (Fig. 1(f)), detected in the supernatant, was also labeled with biotin, suggesting that this secreted Klotho, at least in part, was once present on the cell surface membrane and then secreted from the cell surface to supernatant, because tissues were biotinylated shortly, washed out thoroughly and then cultured. We next introduced *klotho* and *klotho*-EGFP expression plasmids into CHO cells, respectively. Distinct dual bands, corresponding to the products of the transfected cDNAs, were observed in the cell lysates, whereas the both supernatants commonly gave a 130 kDa band (Fig. 1(h)). Additionally, in immunodetection with anti-GFP antisera, the EGFP domain that fused with C-terminus of Klotho was not detectable in the supernatant of Klotho-EGFP expressing cells (Fig. 1(i)), suggesting that the 130 kDa Klotho detected in serum and CSF should be cleaved from cell membrane.

3.4. Glycosylation of Klotho protein alters its relative mobility

As shown in Fig. 2(a), Mink1 mAb precipitated two bands of 135 and 120 kDa from the lysate of CHOTM2 and a single band of 130 kDa from the culture media of the same cells, while KM2119 mAb precipitated only 120 kDa band from the cell lysate. Treated with *N*-glycosidase, both immunoprecipitants from cell lysate revealed a unique band of about 110 kDa that is consistent with the predicted molecular weight from amino acid residues, whereas the product of secreted Klotho protein treated with *N*-glycosidase gave a single band with a little accelerated mobility than those of membrane-bound Klotho proteins. This phenomenon was also confirmed by the addition of tunicamycin, an inhibitor of N-glycosylation, to the culture (Fig. 2(b)). These data suggested that the difference

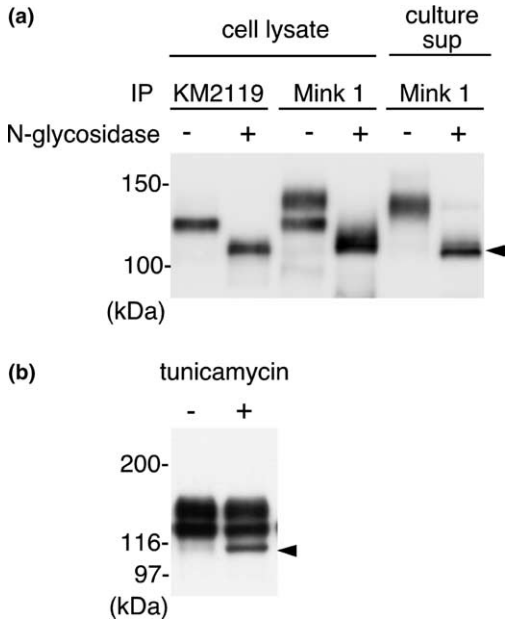

Fig. 2. Post-translational modification of Klotho by N-glycosylation. (a) Klotho was immunoprecipitated with Mink1 or KM2119-conjugated beads from lysates or culture media of CHOTM2. The products were then treated with (+) or without (−) *N*-glycosidase for 30 min at 37 °C, and applied for immunoblotting with KM2076. (b) CHOTM2 cells were treated with (+) or without (−)1 μg/ml tunicamycin for 90 min at 37 °C, and lysed for immunoblot analysis with KM2076.

in molecular weight of the bands from cell lysate depended on the different attached sugar moieties and the secreted Klotho protein was glycosylated as well as membrane-bound Klotho.

3.5. Oligomerized formation of Klotho

Since β-glycosidase family members have been suggested to have a tendency to dimerize, we examined the association of Klotho proteins by size exclusive gel filtration. Secreted Klotho protein was estimated to be between 158 and 670 kDa by comparison with molecular weight markers, suggesting that Klotho was oligomerized or bound to the other protein(s) (Fig. 3(a)). To examine the possibility of self-association of klotho protein, Cos-7 cells expressing Klotho-HA, Klotho-Flag or both were lysed and coimmunoprecipitated with either anti-HA or anti-Flag antibody-conjugated beads. As a result, anti-Flag antibody-conjugated beads could immunoprecipitate HA-tagged Klotho and conversely, anti-HA antibody-conjugated beads also could immunoprecipitate Flag-tagged Klotho (Fig. 3(b)). These data support the hypothesis that Klotho protein should self-associate at least in the cells and be oligomerized as a unique molecular complex in the extracellular fluids.

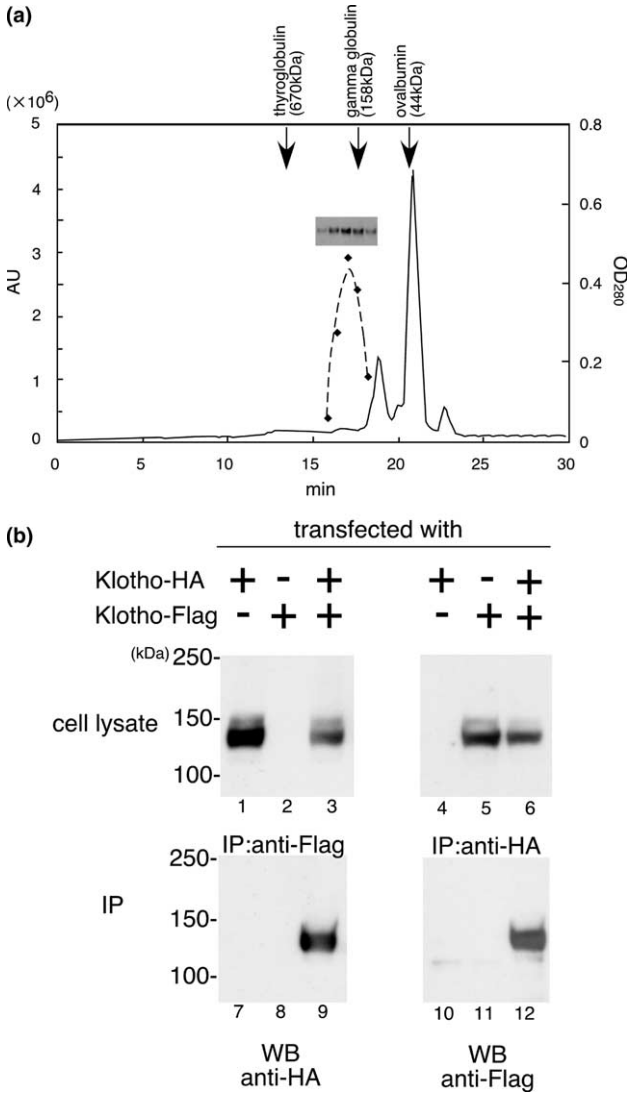

Fig. 3. Oligomerized formation of Klotho. The concentrated supernatant of CHOTM2 was separated through a size exclusive gel filtration system. Absorbance of UV (280 nm) was monitored and blotted as a solid line. The positions of three marker proteins used for molecular weight standards are indicated. Collected fraction samples were subjected to immunoblotting and the amounts of Klotho protein were measured and blotted as a dotted line (a). Cos-7 cells were transfected with expression vectors for HA-tagged (pcDNA3-Klotho-HA) and/or Flag-tagged (pcDNA3-Klotho-Flag) Klotho. After a 48 h incubation, cell lysates (lanes 1–6) and immunoprecipitants with M2, an anti-Flag antibody (lanes 7–9) and an anti-HA antibody (lanes 10–12) were analyzed by Western blotting (b).

4. Discussion

Despite our attempts, we could not detect soluble KL1, the product of alternatively spliced *klotho* mRNA in extracellular compartments. mRNA encoding soluble KL1 is very low in mice but apparently considerable in humans [6,7]. Thus, at least in humans, detectable amounts of KL1 may possibly be produced and secreted, but then might be rapidly eliminated to undetectable levels in serum. On the contrary, 130 kDa Klotho proteins are found in both sera and CSF. Previous failure in detection of the secreted Klotho may be due to the potency of the original antibodies used, because KM2076 and KM2119 react preferentially to 120 kDa Klotho, suggested as a intracellular form, in IP method (Table 1).

Previous studies suggested that humoral factor(s) might mediate the biological function of Klotho, at least in part and its deficiency might be involved in the unique state of klotho mutant mice [1,3–5]. In these examinations, the full-length *klotho* cDNA was always utilized and shown to be effective for the rescue of mutant phenotypes, whereas the ectopic expression of the spliced variant *klotho* cDNA encoding only KL1 was not

effective (Mori et al., unpublished observations). The accumulating evidences suggest a possible importance of the products of full-length *klotho* cDNA and it is possible to propose a revised hypothesis that the secreted 130 kDa Klotho may play an important role in vivo. For the functional evaluation of the secreted Klotho, it is critical to distinguish the humoral action of the secreted Klotho itself from the humoral events resulting from the cell-autonomous function of membrane-bound Klotho.

Nonetheless, the type of events that can induce the release of Klotho, the extent of its range, or where it accumulates in vivo, have yet to be addressed. The determination of the exact cleavage site and the identification of the cleavage enzyme remain to be solved. It is also necessary to study the role(s) of the secreted Klotho in circulating CSF. This is in addition to the evidence gathered for the cell autonomous actions of the membrane Klotho. Future studies thus remain to resolve the distinct physiological roles to which the secreted and membrane forms of Klotho proteins contribute in vivo.

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