Regulation of Renal Outer Medullary Potassium Channel and Renal K⁺ Excretion by Klotho

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ABSTRACT

Klotho is an aging-suppression protein predominantly expressed in kidney, parathyroid glands, and choroids plexus of the brain. The extracellular domain of Klotho, a type-1 membrane protein, is secreted into urine and blood and may function as an endocrine or paracrine hormone. The functional role of Klotho in the kidney remains largely unknown. Recent studies reported that treatment by the extracellular domain of Klotho (KLe) increases cell-surface abundance of transient receptor potential vanilloid type isoform 5, an epithelial Ca²⁺ channel critical for Ca²⁺ reabsorption in the kidney. Whether Klotho regulates surface expression of other channels in the kidney is not known. Here, we report that KLe treatment in-

creases the cell-membrane abundance of the renal K⁺ channel renal outer medullary potassium channel 1 (ROMK1) by removing terminal sialic acids from *N*-glycan of the channel. Removal of sialic acids exposes underlying disaccharide galactose-*N*-acetylglucosamine, a ligand for a ubiquitous galactoside-binding lectin galectin-1. Binding to galectin-1 at the extracellular surface prevents clathrin-mediated endocytosis of ROMK1 and leads to accumulation of functional channel on the plasma membrane. Intravenous administration of KLe increases the level of Klotho in urine and increases urinary excretion of K⁺. These results suggest that Klotho may have a broader function in the regulation of ion transport in the kidney.

Mice homozygous for hypomorphic insertional mutation in the *Klotho* gene exhibit multiple phenotypes closely resembling human aging, including shortened life span, muscle and skin atrophy, pulmonary emphysema, osteopenia, hyperphosphatemia, and vascular and soft tissue calcification (Kuro-o et al., 1997). The encoded protein, Klotho, is a type-1 single-pass membrane protein with a large extracellular domain, a membrane-spanning segment, and a short (~20

amino acids) intracellular carboxyl terminus (Kuro-o et al., 1997). Overexpression of Klotho extends life span in mice, supporting that Klotho is an aging-suppression molecule (Kurosu et al., 2005).

The biological function of Klotho and how Klotho deficiency causes aging remain elusive. Klotho is predominantly expressed in distal renal tubules of the kidney, parathyroid glands, and choroids plexus of the brain (Kuro-o et al., 1997; Kurosu et al., 2005; Imura et al., 2007). The large extracellular domain of Klotho is cleaved by a membrane-anchored protease ADAM10 and secreted into blood, urine, and cerebrospinal fluid (Imura et al., 2004; Kurosu et al., 2005; Chen et al., 2007). The presence of the extracellular domain of Klotho in blood, urine, and cerebrospinal fluid suggests that it may function as an endocrine or paracrine hormone. Recent studies have revealed distinct biological functions for membrane Klotho and the extracellular domain of Klotho

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ABBREVIATIONS: FGF, fibroblast growth factor; KLe, extracellular domain of Klotho; KL1 and KL2, repeat 1 and 2 of the extracellular domain of Klotho; ROMK1, renal outer medullary potassium channel 1; TRPV5, transient receptor potential vanilloid type isoform 5; HEK, human embryonic kidney; CHO, Chinese hamster ovary; GFP, green fluorescent protein; ST6, α 2,6-sialyltransferases; ST6Gal-1, galactosyl- α 2,6-sialyltransferase isoform-1; ST3Gal-1, galactosyl- α 2,3-sialyltransferase isoform-1; CHC, clathrin heavy chain; Cav-1, caveolin-1; DANA, 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid; GnT-V, *N*-acetylglucosaminyltransferase V; LacNAc, *N*-acetyllactosamine; GlcNAc, *N*-acetylglucosamine; SNA, *Sambucus nigra* agglutinin; siRNA, small interference RNA; PBS, phosphate-buffered saline; DN, dominant-negative; DN DII, dominant-negative dynamin II; WT DII, wild-type dynamin II.

(i.e., soluble Klotho) (Kurosu and Kuro-o, 2008). Both full-length membrane Klotho and soluble extracellular domain of Klotho bind to multiple fibroblast growth factor (FGF) receptors and increase their affinity for FGF23 (Kurosu et al., 2006; Urakawa et al., 2006). FGF23 is a circulating hormone that decreases serum phosphate levels by suppressing renal phosphate reabsorption (Razzaque and Lanske, 2007). These findings of Klotho as an obligatory receptor for FGF23 may explain why *Klotho*($-/-$) mice and *Fgf23*($-/-$) mice exhibit many overlapping phenotypes including hyperphosphatemia (Razzaque and Lanske, 2007).

The extracellular domain of Klotho is composed of two internal repeats, KL1 and KL2, each sharing amino acid sequence homology to family 1 glycosidases (Kuro-o et al., 1997; Ito et al., 2002). Consistent with the idea that Klotho has glycosidase activity, Tohyama et al. (2004) showed that soluble Klotho hydrolyzes β -glucuronides in vitro. More recently, several studies reported that soluble Klotho treatment increases plasma membrane abundance of the renal epithelial Ca^{2+} channel TRPV5 by modifying its *N*-linked glycans (Chang et al., 2005; Cha et al., 2008a). This action of Klotho may underscore the finding that renal tubular Ca^{2+} reabsorption is decreased in Klotho-deficient mice (Tsuruoka et al., 2006).

Whether soluble Klotho regulates other ion channels or membrane glycoproteins by modifying their *N*-glycans is not known. A recent study reported that soluble Klotho has no effect on TRPV4 and TRPM6, two types of channels also present in renal tubules and important for renal ion transport (Lu et al., 2008). In the present study, we investigated the role of soluble Klotho in the regulation of ROMK1, a renal K^{+} channel important for K^{+} secretion. We found that soluble Klotho treatment removes sialic acids from *N*-glycans of ROMK1 and causes retention of the channel on the plasma membrane. Furthermore, intravenous administration of soluble Klotho increases urinary excretion of K^{+} . Thus, soluble Klotho may have a broader function in the regulation of renal ion transport.

Materials and Methods

Molecular Biology, cDNA Constructs, and Preparation of Purified KLe. GFP-tagged TRPV5 and ROMK1 were described previously (Yeh et al., 2005; He et al., 2007). cDNAs for human ST6Gal-1 and ST3Gal-1 (IMAGE Clone; Invitrogen, Carlsbad, CA) were amplified by polymerase chain reaction and subcloned into pEF1 expression vector (Invitrogen). Point mutation was generated by site-directed mutagenesis and confirmed by sequencing. Sequences for sense and antisense small interference RNA (siRNA) oligonucleotides for human galectins and sialyltransferases have been published (Cha et al., 2008a). Production and purification of extracellular domain of murine Klotho [soluble Klotho (KLe)] were as described previously (Kurosu et al., 2005).

Cell Transfection and Whole-Cell Patch-Clamp Recording. Cells [human embryonic kidney (HEK) or Chinese hamster ovary (CHO)] were cultured and transfected with cDNAs encoding GFP-TRPV5 (0.1 μg of DNA per 35-mm dish) or GFP-ROMK1 (0.5 μg) with or without additional constructs (1 μg each) as described previously (Yeh et al., 2005; He et al., 2007). In each experiment, the total amount of DNA for transfection was balanced using an empty vector. For knockdown by siRNA, oligonucleotides (200 nM final concentration each) were mixed with cDNA for ROMK1 for cotransfection. Approximately 24 h after transfection, cells were incubated with Klotho and/or bacterial sialidase for the indicated duration.

Forty to 48 h after transfection, whole-cell currents were recorded by ruptured whole-cell recording using an Axopatch 200B amplifier (Molecular Devices, Sunnyvale, CA) as described previously (Yeh et al., 2005; He et al., 2007). Transfected cells were identified using epifluorescent microscopy. For recording of ROMK1 current, the pipette and bath solution contained 140 mM KCl, 10 mM HEPES, pH 7.4, 2 mM EDTA, and 10 mM HEPES, pH 7.4, and 140 mM KCl, 2 mM MgCl_2 , 2 mM CaCl_2 , 10 mM HEPES, pH 7.4, respectively. For TRPV5 current, the pipette and bath solution contained 140 mM sodium aspartate, 10 mM NaCl, 10 mM EDTA, and 10 mM HEPES, pH 7.4, and 140 mM sodium aspartate, 10 mM NaCl, 1 mM EDTA, and 10 mM HEPES, pH 7.4, respectively. Capacitance and access resistance were monitored and 75% compensated. The voltage protocol consisted of 0-mV holding potential and 400-ms steps from -150 to 100 mV in 25-mV increments. Current density was calculated by dividing current at -100 mV (picoamperes; measured at 25°C) by capacitance (picofarads). Results are shown as mean $\pm$ S.E.M. ($n = 5-10$). Statistical comparisons between two groups of data were made using two-tailed unpaired *t* test. Multiple comparisons were made using one-way analysis of variance followed by *t* test.

Surface Biotinylation Assay. Cells were washed twice with ice-cold phosphate-buffered saline (PBS) and incubated with 0.75 ml of PBS containing 1 mg/ml sulfo-succinimidyl 2-(biotinamido)-ethyl-1,3-dithiopropionate (EZ-Link; Pierce, Rockford, IL) for 30 min at 4°C . After quenching by a Tris-buffered solution (140 mM NaCl, 10 mM Tris/HCl, pH 7.4, and 5 mM KCl), cells were lysed in a radio-immunoprecipitation assay buffer (150 mM NaCl, 50 mM Tris-HCl, pH 7.4, 5 mM EDTA, 1% Triton X-100, 0.5% deoxycholate, and 0.1% SDS) containing protease inhibitor cocktail (Roche, Indianapolis, IN). Biotinylated proteins were precipitated by streptavidin agarose beads (Pierce), heated at 50°C for 3 min, and separated by SDS-polyacrylamide gel electrophoresis for Western blot analysis. ROMK1 proteins were detected by a rabbit polyclonal anti-ROMK1 antibody (Zeng et al., 2002). Each experiment shown was performed at least three times with similar results.

Staining by *Sambucus nigra* Agglutinin and *Maackia amurensis* Agglutinin. CHO cells transfected with indicated cDNA constructs were washed by PBS and fixed by 4% paraformaldehyde (in PBS) for 20 min at room temperature. Thereafter, cells were incubated with biotinylated *S. nigra* agglutinin (SNA) or *M. amurensis* agglutinin (60 $\mu\text{g}/\text{ml}$; Vector Laboratories, Burlingame, CA) for 30 min at 37°C and followed by incubation with Alexa Fluor 594-conjugated streptavidin (1:500 dilution; Invitrogen, Carlsbad, CA) for 30 min at 37°C . Fluorescent differential interference contrast images were obtained using a Nikon Eclipse TE2000-U fluorescent microscope (Nikon, Tokyo, Japan) and overlaid as described previously (Cha et al., 2008a). In each experiment, gain for fluorescence detection was adjusted equally using untransfected cells as a control. To quantify the percentage of transfected cells labeled with SNA before and after KLe treatment (e.g., Fig. 4, B versus D), gain for fluorescence detection was adjusted, such as SNA labeling in cells transfected with GFP + ST6Gal-1 (e.g., Fig. 4F) was below detection.

Animal Studies. Rats (220–250 g body weight; Sprague-Dawley rats from Harlan, Indianapolis, IN) were anesthetized by intraperitoneal injection of Inactin (100 mg/kg b.wt.; Sigma/RBI, Natick, MA). Intravascular volume was maintained by infusion of 0.9% (w/v) NaCl via a jugular vein catheter (0.1 ml/100 g body weight over 4 h). After stabilization, purified KLe (64 pmol in 140 mM NaCl and 10 mM Tris 7.4) was administered intravenously. Urine and blood samples were collected through an indwelling catheter in bladder and through a polyethylene tubing placed in carotid artery at indicated times. Plasma and urine chemistry were analyzed by Core laboratory (University of Texas Southwestern Medical Center at Dallas, Dallas, TX). For Western blot analysis of KLe in urine, fresh urine samples (40 μl) were solubilized in nonreducing sample buffer and fractionated by SDS-polyacrylamide gel electrophoresis, transferred to polyvinylidene difluoride membrane and probed using rat anti-human Klotho monoclonal antibody (Kato et al., 2000). Specific signal was visual-

ized by enhanced chemiluminescence. All animal work was approved by the Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center at Dallas.

Results

Soluble Klotho Increases Cell-Surface Abundance of ROMK1 via *N*-Glycan-Dependent Mechanism. Whether soluble Klotho regulates ion channels other than TRPV5 in the kidney is not known. The extracellular domain of Klotho (KLe) is released into urine and thus may regulate ion channels from the luminal side. In this study, we examine whether soluble Klotho regulates ROMK1, a K^+ channel present in the apical membrane of distal renal tubules that plays an important role in the regulation of renal K^+ secretion (Hebert, 1995). We studied the effect of purified recombinant murine KLe on ROMK1 expressed in HEK cells using ruptured whole-cell patch-clamp recording. As shown, HEK cells transfected with ROMK1 exhibit characteristic inward-rectifying K^+ currents (Fig. 1A). Current density (normalized to capacitance, a measurement of cell surface area) at -100

mV was 495 ± 10 pA/pF in transfected cells (Fig. 1A, $\square$). Control untransfected cells do not display inward-rectifying K^+ currents (7 ± 2 pA/pF at -100 mV; data not shown). Treatment with KLe (100 pM) for ~ 16 –24 h markedly increased ROMK1 current density at -100 mV to 1657 ± 110 pA/pF (Fig. 1A, $\blacksquare$). The concentration of KLe in human urine is estimated at 20 to 200 pM (Cha et al., 2008a). We next examined the duration of KLe treatment required for an increase in ROMK1 current density. HEK cells transfected with ROMK1 were incubated with KLe (100 pM) in the culture medium for the indicated time periods before whole-cell recording. We found that 30-min incubation with KLe did not increase ROMK1 currents (Fig. 1B). Incubation with KLe for ≥ 1 h, however, increased ROMK1 current density (Fig. 1B).

Treatment with KLe increases cell-surface abundance of TRPV5 by modifying its *N*-glycans (Cha et al., 2008a). ROMK1 is *N*-glycosylated at a single *N*-glycosylation site, asparagine-117 of ROMK1 (Schwalbe et al., 1998). We next examined whether the effect of KLe to increase ROMK1

Fig. 1. The effect of soluble Klotho on ROMK1. **A**, soluble Klotho increases whole-cell current density of wild-type ROMK1. HEK cells were transfected with GFP-tagged wild-type ROMK1. Twenty-four hours after transfection, cells were incubated with purified KLe (100 pM) for 16 to 24 h before whole-cell recordings. Whole-cell current density (currents normalized to the cell surface area) versus voltage was measured and showed characteristic inwardly rectifying ROMK1 currents in cells treated with KLe ($\blacksquare$) or without KLe ($\square$). **B**, time course of KLe treatment on ROMK1. Bar graph shows current density (pA/pF) at -100 mV. Cells expressing ROMK1 were incubated with KLe for the indicated duration before ruptured whole-cell recording. Cells were incubated with KLe added to culture media (at 37°C). $^*p < 0.05$ versus vehicle (no KLe; $\square$). **C**, effect of KLe on ROMK1 lacking asparagine-117. Cells were transfected with asparagine-117 to glutamine ROMK1 mutant (N117Q) and treated with ($\blacksquare$) or without ($\square$) KLe (200 pM) for 24 h. **D**, effect of bacterial sialidase on ROMK1. The effect of sialidase (Siad) was not additive to that of KLe (200 pM). Current density (pA/pF; at -100 mV) is shown. $^*p < 0.05$ versus vehicle (no KLe). **E**, effects of KLe and bacterial sialidase on surface abundance of ROMK1. "Gly" indicates glycosylated ROMK1. DANA is a specific sialidase inhibitor. DANA ($0.1 \mu\text{M}$) was added to cells simultaneously with KLe (200 pM). Treatment by KLe or Siad did not cause a detectable shift in migration (in low-exposure films, data not shown). This is consistent with the idea that only terminal sialic acids are removed. **F**, effect of KL1 and KL2 repeat on ROMK1. The effect of KLe (100 pM) on ROMK1 is greater than the individual effect of KL1 and KL2 (100 pM each), respectively. $^*p < 0.05$ versus control (CTRL).

current density is dependent on its *N*-glycans. Cells were transfected by wild-type ROMK1 or mutant ROMK1 that carry asparagine-117 to glutamine mutation (N117Q). We found that treatment with KLe did not increase current density of N117Q ROMK1 mutant (Fig. 1C). We have found that the extracellular domain of Klotho functions as a putative sialidase to remove terminal sialic acids from *N*-glycans of TRPV5 (Cha et al., 2008a). To investigate the role of removal of sialic acids in mediating the increase of ROMK1, we examined the effect of sialidase from *Clostridium perfringens* on ROMK1 and found that it increased the current density of ROMK1 (Fig. 1D). The effects of bacterial sialidase and KLe on ROMK1 were not additive (Fig. 1D). Moreover, preincubation with a specific inhibitor of sialidase, 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid (DANA) (Usuki et al., 1988; Cha et al., 2008a), prevented the increase in ROMK1 current density by KLe or by the bacterial sialidase (data not shown).

We further examined the effect of KLe on surface abundance of ROMK1 using biotinylation assays. As shown in Fig. 1E, cell-surface biotinylated ROMK1 proteins consist of two major bands: one is probably glycosylated (indicated by "Gly"), and the other is an underglycosylated form. This is probably due to the fact that ROMK1 is tetrameric, and glycosylation of subunits is heterogeneous. Consistent with the results of whole-cell current density, KLe treatment increased the surface abundance of ROMK1, and preincubation with DANA prevented this effect (Fig. 1E). The bacterial sialidase also increased surface abundance of ROMK1, and the effects of bacterial sialidase and KLe were not additive (Fig. 1E). Together, these results are consistent with the hypothesis that soluble Klotho controls surface expression of ROMK1 via removal of sialic acids from glycan chains.

The extracellular domain of Klotho is composed of two

internal repeats, KL1 and KL2, each sharing amino acid sequence homology to family 1 glycosidases (Kuro-o et al., 1997; Ito et al., 2002). We examined whether each KL repeat can regulate ROMK1 independently. We found that purified KL1 and KL2 domain of Klotho (100 pM each) each independently increased ROMK1 current density (Fig. 1F). At the equal concentration (100 pM), the effect of the full extracellular region ("KLe") on ROMK1 current density is greater than the effect of each individual domain on ROMK1 current density (Fig. 1F), consistent with the fact that KL1 and KL2 each can regulate ROMK1. Similar to the effect of KLe, the effects of KL1 and KL2 were prevented by DANA (data not shown).

Soluble Klotho Increases Surface Abundance of ROMK1 by Decreasing Clathrin-Dependent Endocytosis of the Channel. Soluble Klotho treatment increases cell-surface abundance of TRPV5 by delaying its endocytosis (Cha et al., 2008a). Endocytosis of TRPV5 occurs via caveolae-mediated pathway (Cha et al., 2008b). In contrast, ROMK1 undergoes constitutive endocytosis via clathrin-coated vesicles (Zeng et al., 2002). Thus, it is interesting to ask whether soluble Klotho treatment increases surface abundance of ROMK1 by affecting its endocytosis. To examine this question, we first used a dominant-negative (DN) dynamin II (K44A rat dynamin II) to disrupt endocytosis of ROMK1. We found that coexpression with DN dynamin II (DN DII) increased baseline ROMK1 current (Fig. 2A), confirming that ROMK1 undergoes constitutive endocytosis. Moreover, coexpression with DN dynamin II prevented an increase of ROMK1 current density by KLe (Fig. 2A). For comparison, coexpression with wild-type dynamin II (WT DII) did not prevent the increase in ROMK1 current density by KLe (Fig. 2A).

Both clathrin-coated vesicles- or via caveolae-mediated en-

Fig. 2. Increase in ROMK1 current density by soluble Klotho is dependent on clathrin-mediated endocytosis. **A**, coexpression with DN DII prevents increase of ROMK1 by KLe. Cells were transfected with WT DII or K44A mutant DN DII and treated with KLe (100 pM) for 24 h. *, $p < 0.05$ versus vehicle (-KLe; □). **B**, cells were transfected with ROMK1 plus control oligonucleotides (CTR), siRNA for CHC or siRNA for Cav-1 and treated with KLe (100 pM) for 24 h. *, $p < 0.05$ versus vehicle (-KLe; □). **C**, WT cells or caveolin-1-null cells [Cav-1(-/-)] were transfected with ROMK1 and treated with KLe (100 pM) for 24 h. *, $p < 0.05$ versus vehicle (-KLe; □). **D**, WT cells or Cav-1(-/-) were transfected with TRPV5 and treated with KLe (100 pM) for 24 h. Insets, TRPV5 current density in caveolin-1-null cells cotransfected with empty vector or with recombinant caveolin-1. *, $p < 0.05$ versus vehicle (-KLe; □).

docytosis require dynamin (Conner and Schmid, 2003). To further distinguish between the role of clathrin-coated vesicles versus caveolae in endocytosis of ROMK1 and the effect of KLe, we used siRNA to knock down clathrin heavy chain (CHC) or caveolin-1 (Cav-1). Caveolin-1 is an essential component of caveolae (Cohen et al., 2004). We found that knockdown of CHC but not of Cav-1 prevented an increase in ROMK1 current density by KLe (Fig. 2B). For comparison, control oligonucleotides (CTR) did not prevent KLe-mediated increase in ROMK1 current density (Fig. 2B). To further confirm that endocytosis of ROMK1 is not via caveolae and that the effect of soluble Klotho is not specific for caveolae-mediated endocytosis, we made use of a cell line derived from caveolin-1 knockout mice [Cav-1(−/−) cells]. We found that KLe increased ROMK currents expressed in Cav-1(−/−) cells and in WT cells (Fig. 2C). In contrast, KLe did not increase TRPV5 current density expressed in Cav-1(−/−) cells (Fig. 2D). Forced expression of recombinant caveolin-1 rescued the ability of Klotho to increase TRPV5 currents in Cav-1(−/−) cells (Fig. 2D and inset). These results are consistent with the idea that TRPV5 is internalized via caveolae and that Klotho works by preventing caveolae-mediated endocytosis of TRPV5. Together, these results support the idea that soluble Klotho decreases clathrin-dependent retrieval of ROMK1, allowing them to accumulate at the cell surface. Thus, the effect of soluble Klotho to increase cell surface abundance of ion channels occurs independently of pathways involved in their internalization.

Soluble Klotho Specifically Targets α 2,6-Linked Sialic Acid of *N*-Glycans of ROMK1. We have found that soluble Klotho specifically targets α 2,6-sialic acid on the *N*-glycan of TRPV5 (Cha et al., 2008a). Here, we further examined whether soluble Klotho targets α 2,6-sialic acid on the *N*-glycan of ROMK1. To examine this question, we altered sialic acid residues on the *N*-glycan of ROMK1 by knocking down enzymes that are involved in their synthesis. Normally, sialic acids can be linked to underlying sugar residues via α 2,3, α 2,6, or α 2,8 glycosidic bond. Each linkage is catalyzed by a specific type of sialyltransferases, named ST3, ST6, and ST8 for α 2,3-, α 2,6-, or α 2,8-sialyltransferase, respectively (Yasukawa et al., 2005; Patel and Balaji, 2006). There are five members of ST6s in humans, named ST6Gal-1, ST6Gal-2, and ST6GalNAc-1, -2, and -4, respectively (Yasukawa et al., 2005; Patel and Balaji, 2006). ST6Gal-1 and -2 catalyze the transfer of sialic acids via α 2–6-linkage to the underlying galactose residues of *N*-linked glycoproteins (Patel and Balaji, 2006). ST6GalNAc-1, -2, and -4 catalyze the transfer of sialic acids via α 2–6-linkage to *N*-acetylgalactosamine residues of *O*-linked glycoproteins. ST6GalNAc-3 does not exist in humans. ST6Gal-1 and ST6Gal-2 are specific for *N*-linked glycan chains, suggesting that they may be enzymes involved in the synthesis of sialic acids on ROMK1. To examine whether soluble Klotho targets α 2,6-sialic acid on the *N*-glycan of ROMK1, we carried out siRNA knockdown of ST6Gal-1, ST6Gal-2, or ST6GalNAc-1, -2, and -4. We found that knockdown of ST6Gal-1, but not ST6Gal-2 or ST6GalNAc-1, -2, and -4 combined (indicated as “other ST6s”), prevented the effect of KLe on ROMK1 (Fig. 3A), indicating that ST6Gal-1 is responsible for the synthesis of sialic acid substrate for soluble Klotho.

CHO cells contain endogenous ST3s but not ST6s (Fukuta et al., 2000). We found that KLe had no effects on ROMK1

expressed in CHO cells (Fig. 3B). Forced expression of recombinant ST6Gal-1 but not ST3Gal-1 conferred the regulation of ROMK1 by KLe in CHO cells (Fig. 3B). Thus, soluble Klotho regulates ROMK1 only when they are sialylated via α 2,6-linkage. ST6Gal-1, which is ubiquitously expressed in human tissues (Yasukawa et al., 2005), is responsible for the synthesis of α 2,6-sialic acid on ROMK1.

Figure 3C shows a typical complex type *N*-linked glycan that may be present in cell membrane glycoproteins, such as ROMK1. There are four potential antennary branches distinguished by β 2 or β 4 linkage to an underlying α 3-linked mannose or β 2 or β 6 linkage to an underlying α 6-linked mannose (Schauer, 1991). The Golgi enzyme *N*-acetylglucosaminyltransferase V (GnT-V) catalyzes the addition of β 1,6-*N*-acetylglucosamine [GlcNAc β (1,6)] to the α 6-mannose (Demetriou et al., 2001). This will lead to formation of a tri (2,2,6) or tetra (2,4,2,6) antennary glycan depending on whether the β 4 branch on α 3-mannose is formed (Demetriou et al., 2001) (Fig. 3C). The GlcNAc β (1,6) branch is the preferred substrate for the addition of poly(*N*-acetylglucosamine) (LacNAc). The affinity of polymeric LacNAc for binding to ligands such as galectin-1 (see below) is much stronger than that of LacNAc monomer (Leppänen et al., 2005). We there-

Fig. 3. α 2,6-sialic acids are the target for soluble Klotho. **A**, effect of knockdown of ST6Gal-1 but not ST6Gal-2 or other ST6 sialyltransferases on KLe-mediated increase of ROMK1. HEK cells were transfected with ROMK1 mixed with control oligonucleotide (CTRL), siRNA for ST6Gal-1, siRNA for ST6Gal-2, or pooled siRNAs for other ST6s and treated with KLe (100 pM) for 24 h. *, $p < 0.05$ versus vehicle (−KLe; □). **B**, effect of KLe on ROMK1 expressed in CHO cells. CHO cells were transfected with ROMK1 plus empty vector, ST3Gal-1, or ST6Gal-1 and treated with KLe for 24 h. *, $p < 0.05$ versus vehicle (−KLe; □). **C**, typical complex-type tetra-antennary *N*-glycan present in mammalian cell surface glycoproteins. GnT-V catalyzes the addition of GlcNAc β (1,6) to the α 6-mannose. The GlcNAc β (1,6) branch is preferred substrate for addition of poly(LacNAc). **D**, soluble Klotho increased ROMK1 current density in Lec4 cells expressing both ST6Gal-1 and GnT-V but not in Lec4 cells expressing only ST6Gal-1 or only GnT-V. In each, *, $p < 0.05$ versus vehicle control (−KLe; □). Because interference of *N*-glycan synthesis may affect forward trafficking of membrane proteins (Delacour et al., 2006), we analyzed the effect of siRNA knockdown by comparing relative currents.

fore asked whether GlcNAc β (1,6) branch is necessary for the regulation of ROMK1 by soluble Klotho. We investigated this question making use of the Lec4 cell line, a mutant CHO cell line that lacks GnT-V, thus containing no cell surface N-linked GlcNAc β (1,6) branches (Chaney et al., 1989). We found that KLe treatment increased current density of ROMK1 in Lec4 cells cotransfected with ST6Gal-1 and GnT-V but not in Lec4 cells transfected with ST6Gal-1 alone or with GnT-V alone (Fig. 3D). Thus, LacNAc or more likely poly(LacNAc) in the (2,2,6) tri- and/or (2,4,2,6) tetra-antennary N-glycans is necessary for the increase of ROMK1 at the cell surface by soluble Klotho.

Plant lectins are useful tools for studying the structure of glycans because they bind to specific sugar residues (Corfield et al., 1983; Brinkman-Van der Linden et al., 2002). Among them, SNA specifically bind α 2,6-sialylated, but not α 2,3-sialylated, glycoconjugates (Corfield et al., 1983; Brinkman-Van der Linden et al., 2002). We used fluorescent Alexa Fluor 594-attached SNA to examine the effect of soluble Klotho on sialic acid residues on ROMK1. CHO cells were chosen for the study because they do not express endogenous ST6Gal-1 that is responsible for the synthesis of α 2,6-sialic acid target for Klotho. CHO cells were cotransfected with GFP-ROMK1 (Fig. 4, A–D) or GFP (Fig. 4, E–H) plus ST6Gal-1. Fluorescent images were obtained by gating for GFP for detection of transfected cells (Fig. 4, A, C, E, and G, green) or for Alexa Fluor 594 for detection of SNA labeling (Fig. 4, B, D, F, and H, red). We found that in CHO cells cotransfected with GFP-ROMK1 and ST6Gal-1 and without KLe treatment (Fig. 4, A and B), SNA labeling (Fig. 4B, shown in red) was detected in almost every transfected cell (Fig. 4A, shown in green) but not in untransfected cells (cells without green color). These results indicate that expression of ST6Gal-1 produces α 2,6-sialic acids in CHO cells. For comparison, in CHO cells cotransfected GFP-ROMK1 and ST6Gal-1 and treated with KLe (Fig. 4, C and D), reduced SNA labeling (Fig. 4D, red) was observed in transfected cells (Fig. 4B, green). In multiple experiments, we found that the average percentage of transfected cells with SNA labeling were $81 \pm 5\%$ without KLe treatment and $45 \pm 13\%$ with KLe treatment, respectively (mean $\pm$ S.E.M., $n = 3$ for each; $p < 0.03$ without versus with

KLe). Cells transfected with ST3Gal-1, an enzyme that synthesizes α 2,3-sialic acid, did not give rise to SNA labeling (data not shown), confirming the specificity of SNA for α 2,6-sialic acid.

CHO cells cotransfected with GFP and ST6Gal-1 (Fig. 4, E–H) also displayed SNA labeling (Fig. 4, F and H), indicating that recombinant ST6Gal-1 is capable of the synthesis of α 2,6-sialic acids on endogenous glycoproteins. Of note, SNA labeling on endogenous proteins (Fig. 4, F and H) seems to be less than on transfected ROMK1 (Fig. 4A). This is probably due to the fact that, in the absence of ST6Gal-1, its potential substrates are capped by other enzymes (Martin et al., 2002). Only transfected ROMK1 channels and a fraction of endogenous glycoproteins synthesized during the expression of ST6Gal-1 would be sialylated via α 2,6-linkage. Alternatively, ROMK1 may be a preferred substrate for ST6Gal-1 compared with endogenous glycoproteins in CHO cells. In contrast to the effect on ROMK1 (Fig. 4, B versus D), soluble Klotho treatment did not affect SNA labeling in cells without ROMK1 (compare Figs. 4F and 4H, without KLe and after KLe treatment, respectively). Thus, soluble Klotho treatment removes α 2,6-sialic acids from glycan chains of ROMK1 but not from the majority of resident surface membrane proteins in CHO cells.

Binding to Galectin-1 Is Essential for Increase in ROMK1 by Soluble Klotho. We next investigated the mechanism by which removal of sialic acids by soluble Klotho leads to increase in cell-surface abundance of ROMK1. As shown in Fig. 3C, sialic acids frequently exist as terminal residues of N-glycan chains. Removal of the terminal sialic acids from N-linked glycans exposes underlying LacNAc (Fig. 3C). LacNAc is a ligand for galectins, a family of galactoside-binding lectins widely present in the animal kingdom (Barondes et al., 1994; Leppänen et al., 2005). Many galectins, including the ubiquitously expressed galectin-1 (Barondes et al., 1994; Leppänen et al., 2005), are secreted and interact with proteins at the extracellular surface. We have found that binding to galectin-1 mediates the effect of soluble Klotho to increase surface abundance of TRPV5 (Cha et al., 2008a). Here, we investigated the role of galectin-1 in the effect of soluble Klotho on ROMK1. Galectin-1, -8, and -9 are expressed in HEK cells (Cha et al., 2008a). We found that knockdown of galectin-1 but not of galectin-8 and -9 prevented the increase of ROMK1 by KLe (Fig. 5A). To confirm the role of galectin-1 and determine that the role of galectin-1 is via an effect in the extracellular space, we applied an antibody against galectin-1 extracellularly and examined its effect on ROMK1 current density by KLe. We found that extracellular application of an antibody against galectin-1 prevented the increase of ROMK1 by KLe (Fig. 5B). As a control, extracellular application of an antibody against galectin-8 did not prevent the effect of KLe to increase ROMK1 current density. These results support that galectin-1 working in the extracellular space is necessary for an increase in ROMK1 current density by soluble Klotho.

These results do not address whether this effect of galectin-1 is due to a direct binding with LacNAc. To answer this question, we used LacNAc to compete for binding of desialylated N-glycans of ROMK1 to galectin-1. We found that extracellular application of LacNAc (labeled “LN” in Fig. 5C) prevented the increase of ROMK1 current density by KLe. It is known that sialylation of LacNAc at the α 2,6 position but

Fig. 4. Treatment by soluble Klotho prevents binding of SNA to ROMK1. A to D, CHO cells transfected with GFP-ROMK1 plus ST6Gal-1. Cells were treated with KLe (500 pM) for 1 h (A and B) or without KLe (C and D). After washing off KLe, cells were incubated with biotinylated SNA (B and D), followed by Alexa Fluor 594-labeled streptavidin. E to H, CHO cells were transfected with GFP plus ST6Gal-1. Cells were treated with KLe (E and F) or without KLe (G and H). Thereafter, cells were incubated with biotinylated SNA (F and H), followed by Alexa Fluor 594-labeled streptavidin. In each experimental condition, fluorescent images were acquired by gating for GFP fluorescence (A, C, E, and G) and for Alexa Fluor 594 fluorescence (B, D, F, and H) to detect transfected cells and labeling by SNA, respectively.

not at the $\alpha 2,3$ position interferes with its binding to galectin-1 (Leppänen et al., 2005). For comparison, we found that extracellular application of $\alpha 2,3$ -sialylated LacNAc (labeled “3LN” in Fig. 5C) but not of $\alpha 2,6$ -sialylated LacNAc (“6LN” in Fig. 5C) prevented KLe effect on ROMK1. These results support the idea that removal of terminal sialic acids from *N*-glycan of ROMK1 by Klotho exposes underlying LacNAc, allowing it to bind to galectin-1. Binding of ROMK1 (via LacNAc) to galectin-1 prevents endocytosis of ROMK1 and leads to accumulation on the cell surface. It should be mentioned that the lack of binding between galectin-1 and $\alpha 2,6$ -sialylated LacNAc explains why removal of $\alpha 2,6$ -sialic acids from *N*-glycans of ROMK1 by soluble Klotho is required for the increase in surface abundance.

Administration of KLe Increases Urinary K^+ Excretion in Rats. ROMK1 plays an important role in the regulation of K^+ secretion in the distal nephron (Hebert, 1995). We next investigated the physiological role of soluble Klotho in the regulation of K^+ secretion in rats. We found that urinary K^+ excretion rate was $0.41 \pm 0.07 \mu\text{Eq/min}$ at baseline (i.e., before KLe) and $1.09 \pm 0.25 \mu\text{Eq/min}$ (not significantly different versus vehicle) and $1.60 \pm 0.26 \mu\text{Eq/min}$ ($p < 0.05$ versus vehicle) at 1 and 2 h after intravenous administration of KLe, respectively (Fig. 6A, ●). As a time control, vehicle was administered, and urinary K^+ excretion was 0.44 ± 0.10 , 0.57 ± 0.05 , and $0.58 \pm 0.09 \mu\text{Eq/min}$ at baseline (before vehicle), 1, and 2 h after intravenous administration of vehicle, respectively (Fig. 6A, ○). The fractional excretion of K^+ was also increased by intravenous administration of KLe ($p < 0.01$, 2 h after Klotho versus baseline, Fig. 6B). Interestingly, plasma K^+ concentrations were not significantly altered by soluble Klotho (Fig. 6C, not significantly different, 1 and 2 h versus baseline for KLe-treated group and for vehicle group). The stable plasma K^+ concentration, despite the large increase in renal K^+ excretion in rats that

Fig. 5. Binding with galectin-1 is critical for the increase of ROMK1 by soluble Klotho. A, HEK cells were transfected with ROMK1 plus control oligonucleotide (CTRL) or siRNA oligonucleotides for the indicated galectin and treated with KLe (100 pM) for 24 h. *, $p < 0.05$ versus before KLe (□). B, antibody against galectin-1 (GLTN-1), but not against galectin-8 (GLTN-8), prevents the increase of ROMK1 by KLe. Transfected cells were incubated with KLe and indicated antibody (15 nM). C, LacNAc (LN) and $\alpha 2,3$ -sialylated LacNAc (3SLN) but not $\alpha 2,6$ -sialylated LacNAc (6SLN) prevents the increase of ROMK1 by KLe.

received intravenous soluble Klotho, may be because of the fact that soluble Klotho antagonizes the action of insulin on the peripheral tissues, thus causing redistribution of intracellular K^+ to plasma (Kurosu et al., 2005). Thus, soluble Klotho increases urinary K^+ excretion by enhancing net tubular K^+ secretion. We found that epitope-tagged KLe appeared in urine (with an estimated concentration of ~ 100 pM) at 2 h after intravenous administration (data not shown). These results support the thesis that administered KLe regulates ROMK1 channels from the luminal side.

Discussion

Klotho is an aging-suppression molecule that is abundantly expressed in the distal tubules of kidney and in several other tissues (Kuro-o et al., 1997). It exists in two different forms: a membrane form, and a soluble form equal to the shed extracellular domain (Kurosu and Kuro-o, 2008). One function of Klotho in kidney is to increase renal phosphate excretion. Klotho does so via dual mechanisms. One mechanism is by suppressing the synthesis of 1,25-vitamin D (Yoshida et al., 2002). The other is by inhibiting renal phosphate reabsorption via sodium phosphate cotransporter NaPi-II (M.-C. Hu and O. Moe, unpublished results). The effect of suppression of 1,25-vitamin D synthesis is believed to be mediated by the membrane form of Klotho (Yoshida et al., 2002). Inhibition of NaPi-II is probably mediated by soluble Klotho (M.-C. Hu and O. Moe, unpublished results). Soluble Klotho also regulates other renal ion transport protein. It stimulates renal Ca^{2+} reabsorption, probably by increasing cell-surface abundance of TRPV5 (Chang et al., 2005; Tsuruoka et al., 2006; Cha et al., 2008a). We now report that soluble Klotho increases cell surface abundance of ROMK1. Soluble Klotho does so by removing $\alpha 2,6$ -specific sialic acids from *N*-glycans of ROMK1 (see model in Fig. 7). Removal of $\alpha 2,6$ -specific sialic acids exposes underlying *N*-acetylglucosamine for binding to galectin-1. Binding to galectin-1 prevents endocytosis of ROMK1 and allows channels to accumulate on the cell surface. Conditions associated with reduced nephron mass, such as chronic renal failure and

Fig. 6. Effect of intravenous administration of KLe on urinary K^+ excretion in rats. A to C, urine and blood samples were collected at baseline (before KLe, indicated as 0 h) and 1 and 2 h after intravenous administration of KLe (●) or vehicle (○). In A and B, *, $p < 0.05$ and < 0.01 KLe versus vehicle, respectively.

aging, have reduced Klotho production in the kidney (Koh et al., 2001). Long-term infusion of angiotensin II down-regulates Klotho expression in the kidney (Mitani et al., 2002). It would be interesting to investigate whether Klotho deficiency contributes to decreased K^+ excretion in old age and in the condition of dietary K^+ restriction (which stimulates the renal angiotensin II system) (Wei et al., 2007).

The mechanism of regulation of ROMK1 by soluble Klotho is similar to that described in our previous report on the regulation of TRPV5 (Cha et al., 2008a). However, it should be noted that ROMK1 and TRPV5 undergo endocytosis via different pathways. ROMK1 is internalized via clathrin-coated vesicles, whereas TRPV5 is internalized via a caveolae-mediated pathway (Zeng et al., 2002; Cha et al., 2008b). For endocytosis via clathrin- and caveolae-mediated endocytosis, target proteins need to be recruited to clathrin-coated pits or caveolin-1-containing lipid rafts. These processes require specific recognition sequence on the target proteins (such as NPXY-like sequence on ROMK1) and interacting partner proteins in the endocytic membrane regions (such as AP2 adaptors for clathrin-coated pits) (Zeng et al., 2002). The findings that endocytosis of both TRPV5 and ROMK1 are delayed suggest that soluble Klotho affects a step proximal to the entry of channels into pathways for endocytosis. We identify this step as binding of channels via *N*-glycan chains to galectin-1.

Similar mechanisms via binding to galectin-3 have been found to be critical for regulating resident time, such as cell surface abundance of cell membrane glycoproteins including receptors for growth-promoting and growth-inhibiting factors and Glut2 transporters (Demetriou et al., 2001; Partridge et al., 2004; Ohtsubo et al., 2005; Lau et al., 2007). In general, membrane receptors for growth-promoting and growth-inhibiting factors have multiple extracellular asparagine residues and thus multiple *N*-glycans. Having multiple *N*-glycan chains is important because the affinity of singular LacNAc for any given galectin is relatively low. It is shown that the number of *N*-glycan chains (that contain LacNAc ligand for galectins) determines the abundance of growth factor receptors at the cell membrane, which is critical for the control of cell growth in response to growth factors (Demetriou et al., 2001; Partridge et al., 2004; Lau et al., 2007). Along this line, it is interesting to note that ROMK1 and TRPV5 each have only one *N*-glycan chain. Nevertheless, both channels exist in tetramers (Hebert, 1995; Hoenderop et

al., 2002). Moreover, we found that removal of sialic acid from the GlcNAc β (1,6) branch is critical for the increase in ROMK1 by Klotho. This branch is the preferred substrate for the addition of polymeric LacNAc, which has a much higher binding affinity for galectin-1 compared with that of monomeric LacNAc (Demetriou et al., 2001). Thus, the tetrameric structure of channels and the presence of polymeric LacNAc probably contribute to strong interaction of channels with galectin-1. Overall, binding between *N*-glycans and galectins is probably an important general mechanism for regulating endocytosis of cell membrane glycoproteins by controlling their entry into clathrin-coated pits or caveolae (Stanley, 2007).

One important question regarding the role of soluble Klotho in the kidney is whether it regulates other ion channels and transporters by modifying their *N*-glycan chain. Recently, Lu et al. (2008) reported that soluble Klotho regulates TRPV5 and its close relative TRPV6, but not TRPV4 and TRPM6, two channels also present in the apical membrane of renal tubules. Our report that soluble Klotho increases cell-surface abundance of ROMK1 in cultured cells and administration of soluble Klotho increases net tubular K^+ secretion in rats suggests that soluble Klotho has a broader functional role in kidney. Whether the lack of regulation of TRPV4 and TRPM6 by soluble Klotho is due to lack of target α 2,6-sialic acid residues is not known. It is presently unknown how injected soluble Klotho reaches urinary space. Nevertheless, the estimated concentration of endogenous soluble Klotho in human urine is between 20 and 200 pM (Cha et al., 2008a), a concentration range we found is sufficient for regulating ROMK1 and TRPV5. This report expands the list of ion channels/transporters potentially regulated by soluble Klotho in the urine to include sodium phosphate transporter NaPi-II, TRPV5, TRPV6, and ROMK1. The list probably will continue to grow. Finally, transgenic overexpression of Klotho ameliorates tubular and glomerular injury in a mouse strain that develops progressive renal injury spontaneously over time (Haruna et al., 2007), supporting a general physiologically beneficial role of Klotho in the kidney.

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Fig. 7. Working model for increase in cell-surface abundance of ROMK1 by soluble Klotho. LacNAc is a ligand for galectin-1. α 2,6-Sialylation of LacNAc prevents its binding to galectin-1. Soluble Klotho remove α 2,6-sialic acids from *N*-glycan chains of ROMK1. See text for further details.

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