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Klotho Up-regulates Renal Calcium Channel Transient Receptor Potential Vanilloid 5 (TRPV5) by Intra- and Extracellular *N*-glycosylation-dependent Mechanisms*

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Background: Klotho is a putative sialidase that modifies the extracellular *N*-glycan of TRPV5 to increase its surface expression.
Results: Intracellular expression of Klotho increased TRPV5 via an *N*-glycosylation-dependent mechanism that requires the sialidase activity of Klotho.
Conclusion: Klotho up-regulates TRPV5 surface abundance by intra- and extracellular mechanisms.
Significance: We investigate a novel function of Klotho to regulate intracellular protein trafficking via enzymatic activity.

The anti-aging protein Klotho is a type 1 membrane protein produced predominantly in the distal convoluted tubule. The ectodomain of Klotho is cleaved and secreted into the urine to regulate several ion channels and transporters. Secreted Klotho (sKL) up-regulates the TRPV5 calcium channel from the cell exterior by removing sialic acids from *N*-glycan of the channel and inhibiting its endocytosis. Because TRPV5 and Klotho coexpress in the distal convoluted tubule, we investigated whether Klotho regulates TRPV5 action from inside the cell. Whole-cell TRPV5-mediated channel activity was recorded in HEK cells coexpressing TRPV5 and sKL or membranous Klotho (mKL). Transfection of sKL, but not mKL, produced detectable Klotho protein in cell culture media. As for sKL, mKL increased TRPV5 current density. The role of sialidase activity of mKL acting inside is supported by findings that mutations of putative sialidase activity sites in sKL and mKL abrogated the regulation of TRPV5 but that the extracellular application of a sialidase inhibitor prevented the regulation of TRPV5 by sKL only. Mechanistically, coexpression with a dominant-negative dynamin II prevented the regulation of TRPV5 by sKL but not by mKL. In contrast, blocking forward trafficking by brefeldin A prevented the effect with mKL but not with sKL. Therefore, Klotho up-regulates TRPV5 from both the inside and outside of cells. The intracellular action of Klotho is likely due to enhanced forward trafficking of channel proteins, whereas the extracellular action is due to inhibition of endocytosis. Both effects involve putative Klotho sialidase activity. These effects of Klotho may play important roles regarding calcium reabsorption in the kidney.

Klotho (KL)[3] was identified serendipitously by Kuro-o *et al.* (1) during experiments to generate a transgenic mouse for a sodium-proton exchanger. Mice homozygous for the hypomorphic *klotho* (*kl*) allele, because of disruption of the promoter region by the transgenic construct, showed multiple phenotypes resembling premature human aging, including a shortened life span, growth retardation, skin atrophy, hypogonadism, hyperphosphatemia, and impaired gait and hearing (2). Conversely, overexpression of Klotho results in an extended life span and increased resistance to oxidative stress (3). Therefore, *kl* was proposed to be an aging suppressor gene (4). In humans, polymorphisms in the *KLOTHO* gene have been reported to be associated with an elevated breast cancer risk, a shortened life span, and coronary artery disease (5–7).

Klotho is predominantly expressed in the renal tubules, parathyroid glands, and choroid plexus of the brain (2). Klotho is a single-pass, type 1 transmembrane glycoprotein encoded by 1012 amino acids. The Klotho protein consists of an N-terminal signal sequence (60 amino acids), two internal repeat sequences known as KL1 and KL2 (each ~440 amino acids), a membrane-spanning domain (21 amino acids), and a short intracellular carboxyl terminus (11 amino acids) (2). The extracellular domain of KL, consisting of the KL1 and KL2 domains (also known as secreted Klotho (sKL)) can be cleaved and released into extracellular fluid such as blood, urine, and cerebrospinal fluid (2, 3, 8–10). Therefore, Klotho exists in two different forms *in vivo*: a full-length membranous form (mKL) and a secreted soluble form (sKL) (11, 12). Secreted Klotho in the systemic circulation originates mostly from cleavage of membranous Klotho rather than from potential alternative splicing

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[3] The abbreviations used are: KL, Klotho; sKL, secreted Klotho; mKL, membranous Klotho; FGFR, FGF receptor; BFA, brefeldin A; DANA, 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid; ER, endoplasmic reticulum.

of the gene (8). Serum levels of secreted Klotho in mice are estimated to be between 50–100 pM (3).

Recent studies have indicated that mKL interacts with FGF receptor (FGFR) and functions as the coreceptor for the ligand FGF23 (13–15). Activation of the mKL and FGFR coreceptor complex by FGF23 negatively regulates body phosphate homeostasis by suppressing 1,25-dihydroxyvitamin D synthesis (therefore decreasing gastrointestinal phosphate absorption) and inhibiting renal phosphate reabsorption (16, 17). In contrast, sKL functions as an exo-/paracrine factor to regulate ion transport and growth factor signaling (3, 18–20).

Klotho inhibits signaling by Wnt, TGF1β, and/or insulin/IGF1 (3, 20–22), and these effects are believed to contribute to the anti-aging function of Klotho. Recent studies have suggested that the regulation of the phosphate metabolism by FGF23 through the mKL and FGFR coreceptor complex plays a critical role in aging suppression. Mice with homozygous knockout of *fgf23* have profound hyperphosphatemia and the same premature aging phenotypes as homozygous *kl* hypomorphic mice (23). Dietary phosphate restriction rescues premature aging and death in both *fgf23* knockout and *kl* hypomorphic mice (24, 25).

In addition to phosphate metabolism, Klotho also regulates renal Ca^{2+} handling. Renal Ca^{2+} excretion is crucial for total body calcium homeostasis and is tightly regulated. About 95–98% of the filtered Ca^{2+} is reabsorbed along renal tubules (26). TRPV5 is a Ca^{2+}-selective channel expressed in the apical membrane of the distal convoluted tubule and connecting tubule that mediates Ca^{2+} entry from ultrafiltrate into cells for transcellular reabsorption (27). Cell surface abundance of TRPV5 is regulated by transcriptional and translational mechanisms, insertion and retrieval of the TRPV5 channel, and negative feedback because of elevated intracellular Ca^{2+} levels (28).

The extracellular KL1 and KL2 domains of Klotho each have a strong amino acid sequence homology to family 1 glycosidases (9). Klotho exhibits some β-glucuronidase activity (29). Chang *et al.* (29) first showed that secreted Klotho regulates cell surface expression of TRPV5 via an *N*-glycan-dependent mechanism. Subsequent studies by Cha *et al.* (30) demonstrated that secreted Klotho exhibits sialidase activity and removes terminal sialic acids from *N*-glycans of TRPV5. Removal of sialic acids exposes the underlying disaccharide galactose-*N*-acetyl-glucosamine for binding to galectin-1 at the extracellular face and, therefore, retention of TRPV5 at the cell surface (30, 31). Because *N*-glycosylation may regulate intracellular protein trafficking (32–34) and because Klotho and TRPV5 coexpress in the distal convoluted tubule and connecting tubule (2, 35), we investigated whether Klotho regulates TRPV5 from the cell interior.

EXPERIMENTAL PROCEDURES

Materials and DNA Constructs—Peroxidase-conjugated rabbit polyclonal anti-GFP antibody was purchased from Invitrogen/Molecular Probes (Eugene, OR), and EZ-Link® sulfo-NHS-SS-biotin and streptavidin-agarose beads were from Thermo Scientific (Rockford, IL). Brefeldin A (BFA) and 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid (DANA) were from Sigma-Aldrich (St. Louis, MO). GFP-tagged TRPV5 contains the coding region of TRPV5 cloned in-frame to a commercial pEGFP-N3 vector (36). Point mutations of secreted and transmembrane Klotho were generated by site-directed mutagenesis (QuikChange kit, Stratagene, La Jolla, CA) and confirmed by sequencing.

Cell Culture and Transfection—HEK293 cells were cultured as described previously (30, 36). Cells were transiently cotransfected with cDNA for the wild-type or *N*-glycan-deficient mutant (N358Q) of GFP-TRPV5 (300 ng per 6-well plate) plus cDNA for membranous KL. Alternatively, the same amounts of wild-type TRPV5 were cotransfected with either wild-type or mutant versions of secreted or membranous KL, respectively. Transfection was performed using Polyfect® reagent (Qiagen, Valencia, CA) according to the protocol of the manufacturer. In each experiment, the total amount of DNA for transfection was balanced by using empty vectors.

Electrophysiological Recording—Approximately 48 h after transfection, cells were dissociated and placed in a chamber for ruptured whole-cell recordings as described previously (24). Transfected cells were identified for recording by green fluorescence. The pipette solution contained 140 mM sodium aspartate, 10 mM NaCl, 10 mM EDTA, and 10 mM HEPES (pH 7.4), and the bath solution contained 140 mM sodium aspartate, 10 mM NaCl, 1 mM EDTA, and 10 mM HEPES (pH 7.4). The resistance of electrodes containing the pipette solution was 1.5–3 MΩ. The cell membrane capacitance and series resistance were monitored and compensated (>75%) electronically using an Axopatch 200B amplifier (Axon Instruments, Foster City, CA). The voltage protocol consisted of a 0 mV holding potential and successive voltage sets (500-ms duration) from −150 to +100 mV in +25 mV increments. Data acquisition was performed using ClampX9.2 software (Axon Instruments). Currents were low pass-filtered at 2 kHz using an eight-pole Bessels filter in the clamp amplifier, sampled every 0.1 ms (10 kHz) with a Digidata-1300 interface, and saved directly to a computer hard drive.

Surface Biotinylation Assay—For biotinylation of cell surface TRPV5, cells were washed with ice-cold PBS and incubated in 0.75 ml of PBS containing 0.75 mg/ml EZ-Link NHS-SS-biotin for 1 h at 4 °C. After being quenched with glycine (100 mM), cells were lysed in radioimmune precipitation assay buffer (150 mM NaCl, 50 mM Tris-HCl, 5 mM EDTA, 1% Triton X-100, 0.5% deoxycholic acid, and 0.1% SDS) containing protease inhibitor mixture (Roche). Biotinylated proteins were precipitated by streptavidin-agarose beads. Beads were subsequently washed four times with PBS containing 1% Triton X-100. Biotin-labeled proteins were eluted in sample buffer, heated at 50 °C for 5 min, separated by SDS-PAGE electrophoresis, and transferred to nitrocellulose membranes for Western blotting. TRPV5 proteins on the membrane were detected using a rabbit polyclonal anti-GFP antibody. Biotinylation experiments were performed three times with similar results.

Data Analysis—Data analysis and curve fitting were performed with Prism (v3.0) software (GraphPad Software, San Diego, CA). Statistical comparisons between two groups of data were made using two-tailed unpaired Student's *t* test. Data are presented as mean ± S.E. (n = 5–10 as indicated).

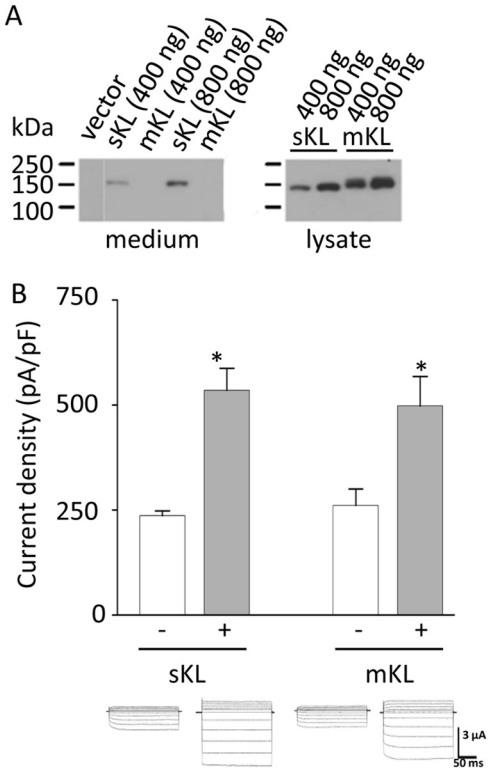

FIGURE 1. **Although it is barely detected in the unconcentrated culture medium of transfected cells, membranous Klotho up-regulates TRPV5 whole-cell current similarly as secreted Klotho.** *A*, an abundance of Klotho in unconcentrated media from cells transfected with 400 or 800 ng of sKL or mKL. Note the abundant expression of mKL relative to sKL in cell lysates. *B*, secreted and membranous Klotho both up-regulate TRPV5 whole-cell current. The *bar graph* shows TRPV5 current density (picoampere/picofarad (*pF*)) at −150 mV cotransfected with control empty vector or plasmids for sKL or mKL. Both sKL and mKL increase the TRPV5 whole-cell current compared with cotransfection with the control ($n = 5$ for each). *, $p < 0.05$. Typical current traces are shown below each specific study group.

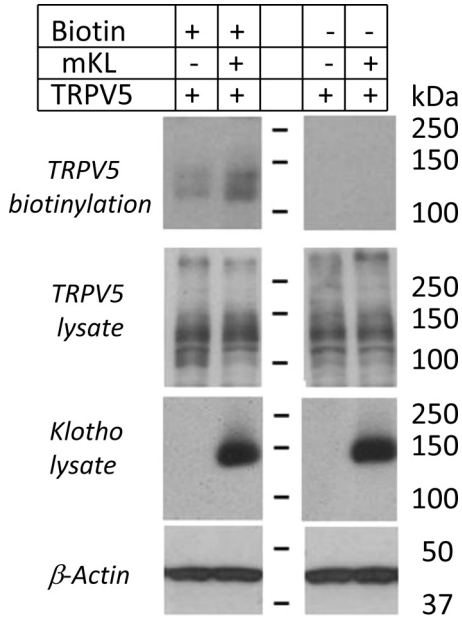

FIGURE 2. **Membranous Klotho increases TRPV5 cell surface abundance.** TRPV5 cell surface abundance measured by biotinylation is increased when cotransfected with mKL compared with the control. TRPV5 abundance at the plasma membrane (*top*) and total lysates (*center*) were analyzed by immunoblot analysis using antibody against GFP. The antibody did not detect a protein signal in untransfected cells (data not shown). The double bands of TRPV5 reflect unglycosylated and glycosylated protein products. For detection of TRPV5 on the surface and in the lysate, 200 and 10 μg of protein were separated by SDS gel electrophoresis, respectively.

RESULTS

Both Membranous and Secreted Klotho Up-regulate TRPV5—To investigate whether Klotho regulates TRPV5 from inside the cell, we designed experiments to compare the effects of full-length membranous Klotho *versus* secreted Klotho on TRPV5. The cDNA construct for secreted Klotho contains nucleotides coding for the signaling peptide and the entire extracellular domain but lacks the transmembrane-spanning and intracellular regions of full-length Klotho. Because the ectodomain of membranous Klotho can be cleaved and secreted into media of cells, we first compared the abundance of Klotho protein in media of cells transfected with either a membranous or secreted Klotho construct. As shown in Fig. 1*A*, Klotho was detected by Western blot analysis in non-concentrated supernatants of media from HEK cells transfected with 400 – 800 ng of DNA coding for secreted Klotho. In contrast, Klotho was not detected in supernatants of cells transfected with the same amount of cDNA coding for membranous Klotho under the same experimental settings. As shown, cotransfection of TRPV5 with 800 ng of DNA coding for either secreted or membranous Klotho caused an increase in TRPV5 current density (Fig. 1*B*). Because much less Klotho, if any, was secreted into media in cells transfected with membranous Klotho, these results support the notion that Klotho can regulate TRPV5 via an intracellular action.

Membranous Klotho Increases TRPV5 Cell Surface Abundance—The increase in TRPV5 current density by secreted Klotho acting from the outside is due to increased cell surface abundance (30). We also examined the effect of membranous Klotho on TRPV5 using a cell surface biotinylation assay. As shown, cotransfection with membranous Klotho resulted in a higher cell surface abundance of TRPV5 compared with the empty vector control (Fig. 2).

Regulation of TRPV5 by Membranous Klotho Requires N-glycosylation of TRPV5—Up-regulation of TRPV5 by secreted Klotho acting from the outside of cells requires *N*-glycosylation on asparagine 358. Mutation of asparagine 358 of TRPV5 to glutamine (N358Q) prevents its regulation by secreted Klotho (19). To determine whether the putative intracellular mechanism of regulation of TRPV5 by Klotho also involves *N*-glycosylation, we examined the effect of membranous Klotho on WT and N358Q TRPV5 mutant channels. We found that cotransfection with membranous Klotho increased the current density of WT TRPV5 but not of the N358Q mutant (Fig. 3*A*), supporting the idea that the intracellular action of Klotho on TRPV5 also requires *N*-glycosylation.

Identification of Critical Residues of Klotho for Up-regulation of TRPV5 via the Putative Sialidase Activity—The KL1 and KL2 domains of Klotho each have a strong amino acid sequence homology to family 1 glycosidases. Family 1 glycosidases contain two highly conserved glutamate residues within the active site known to be critical for enzymatic activity (9, 37). One of these acts as an acid-base catalyst and the other as a nucleo-

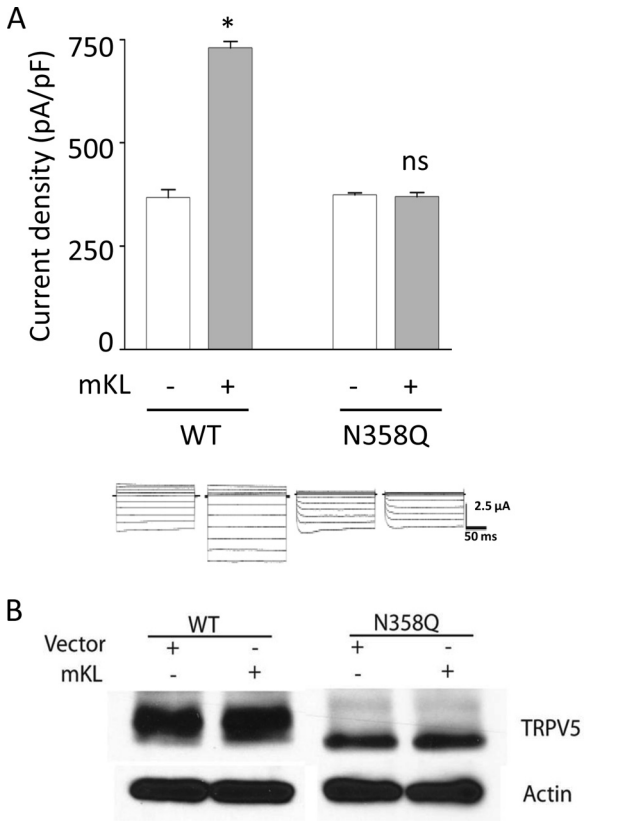

FIGURE 3. **TRPV5 up-regulation by membranous Klotho depends on TRPV5 *N*-glycosylation.** *A*, in contrast to WT TRPV5, no significant increase of TRPV5 whole-cell current was detected when *N*-glycan-deficient N358Q TRPV5 was cotransfected with mKL. The *bar graph* shows the mean ± S.E. ($n = 7$ for each) of TRPV5 current density at −150 mV. *, $p < 0.0001$; *ns*, not significant. *pF*, picofarad. *B*, wild-type TRPV5 and *N*-glycan-deficient N358Q TRPV5 protein densities in total cell lysates do not show any significant difference regarding channel expression with or without mKL cotransfection.

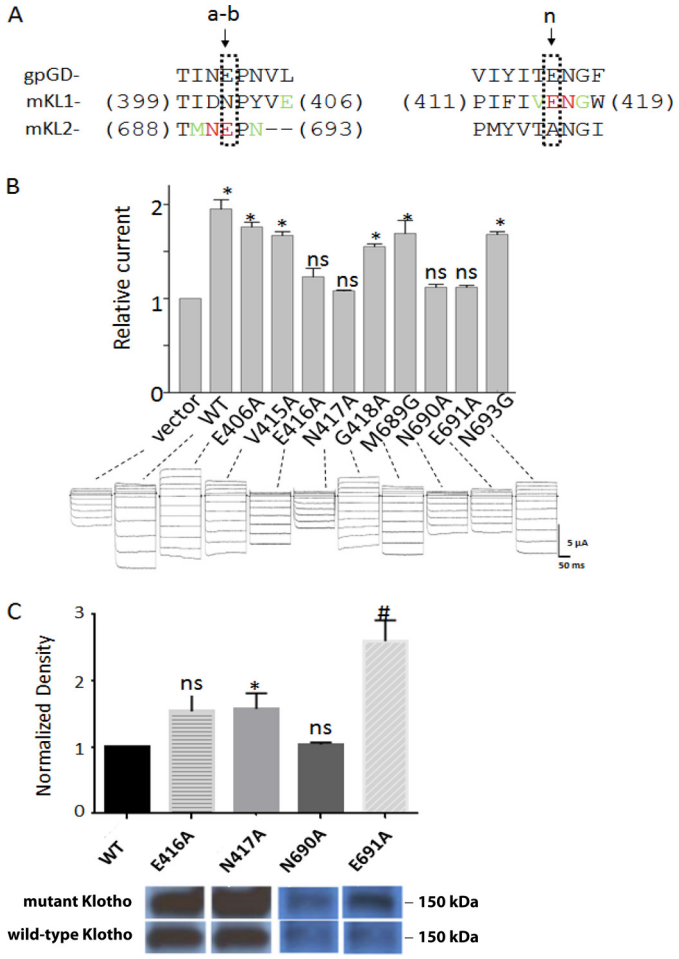

FIGURE 4. **Identification of critical residues of Klotho for up-regulation of TRPV5 via the putative sialidase activity.** *A*, amino acid homology between guinea pig β-glucosidase (*gpGD*) and the mouse Klotho KL1 and KL2 domains (9). Although glutamate is substituted at the acid-base catalyst position (*a-b*) by asparagine in the mKL1 domain, glutamate is conserved in the KL2 domain. At the nucleophile (*n*) position, glutamate is conserved in the mKL1 domain but substituted by alanine (shown) or serine (not shown), respectively. *B*, mutagenesis of evolutionary conserved amino acids positioned around the acid-base catalyst and nucleophile position in secreted Klotho showed that amino acid residues Glu-416, Asn-417, Asn-690 and Glu-691 were crucial for sKL sialidase activity. The *bar graph* shows mean ± S.E. ($n = 5$ for each) of TRPV5 current density at −150 mV. *, $p < 0.005$; *ns*, not significant. Typical current traces are shown below each specific study group. *C*, the lack of TRPV5 stimulation by sKL mutants E416A, N417A, N690A, and E691A is not due to impaired mutant protein expression. Equal volumes of cell culture medium of sKL mutant and wild-type transfected HEK cells were analyzed by immunoblot. Mutant sKL was either secreted to the same degree (no significant difference between WT and A416A or N690A) or even more (N417A and E691A) compared with WT sKL. Average results (mean ± S.E.) from four experiments are shown. The abundance of WT and mutant KL proteins in culture medium was quantified by densitometry (area under the curve). Representative samples of mutant and corresponding wild-type sKL are shown in the *bottom panel*. The secreted KL mutants E416A and N417A were transfected in one experiment, and sKL N690A and E691A were transfected in another experiment, which resulted in two different correlating wild-type KL bands as a positive control. The density of protein abundance was normalized to that in the wild-type KL group (given value of 1). *, $p < 0.05$; #, $p < 0.005$; *ns*, not significant.

phile. In Klotho protein, one of the two glutamate residues within each KL1 and KL2 domain are substituted. Glutamate at the acid-base catalyst position of the KL1 domain is substituted by an asparagine and glutamate at the nucleophile position of the KL2 domain by an alanine or a serine, respectively (Fig. 4*A*). Despite these variations, an alignment of the active site between each KL1 and KL2 repeat and family 1 glycosidases has been proposed (9, 37) (Fig. 4*A*). A previous study by Cha *et al.* (30) has demonstrated that triple mutations of Asn-402 and Glu-416 in the KL1 domain and Glu-691 in the KL2 domain inactivated the ability of Klotho to up-regulate TRPV5. To further test the validity of the alignment and refine the identification of critical residues for the enzymatic activity of Klotho, we performed site-directed mutagenesis of individual amino acids at the corresponding nucleophile and acid-base catalyst positions and surrounding neighbor amino acids.

TRPV5 was cotransfected with empty vector, cDNA encoding WT secreted Klotho, or secreted Klotho carrying mutations of individual amino acids, and TRPV5-mediated current density was measured. Mutant secreted Klotho carrying the mutation of the amino acid Glu-406, Val-415, Gly-418, Met-689, or Asn-693 (Fig. 4*A*, *green*) caused a significant increase in TRPV5 current density compared with empty vector (Fig. 4*B*). In contrast, mutation of Glu-416, Asn-417, Asn-690, or Glu-691 (Fig. 4*A*, *red*) abolished the effect of secreted Klotho to stimulate TRPV5 current density. The lack of stimulation of TRPV5 by the latter four mutants is not due to impaired protein expression (Fig. 4*C*). Furthermore, cotransfection with three times more mutant DNAs (relative to WT secreted Klotho) did not show stimulation of the TRPV5 current (data not shown).

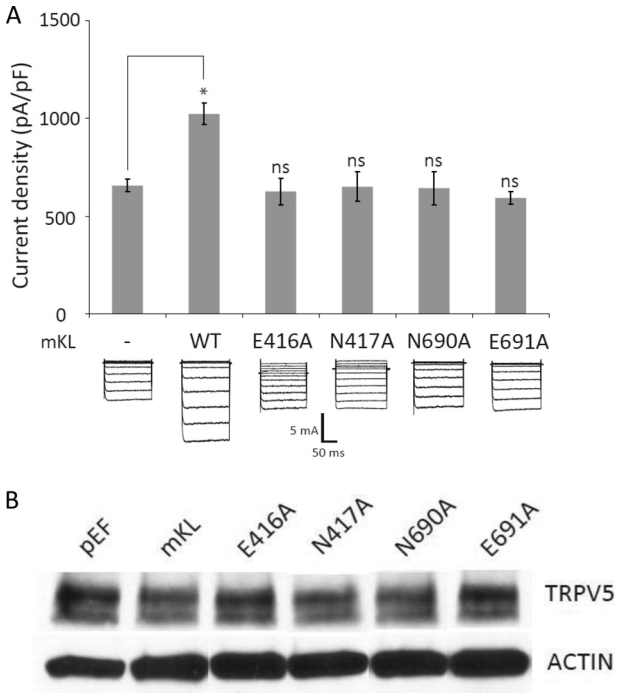

FIGURE 5. **Membranous KL requires sialidase activity for TRPV5 up-regulation.** *A*, we chose the Klotho E416, N417, N690, and E691 residues as crucial amino acid residues for sKL sialidase activity and performed site-directed mutagenesis in membranous KL. In contrast to wild-type membranous KL, the E416A, N417A, N690A, and E691A mutants of membranous KL did not increase TRPV5 current density ($n = 6$ for each). *, $p < 0.0005$; *ns*, not significant. *pF*, picofarad. *B*, TRPV5 protein density in total cell lysate is shown after cotransfection with either the control (*pEF*), wild-type mKL, or the E416A, N417A, N690A, or E691A mKL mutants. No significant difference regarding TRPV5 channel expression was detected. TRPV5 cotransfected with mKL mutants was at least as well expressed in total cell lysate as when cotransfected with mKL wild-type (mKL).

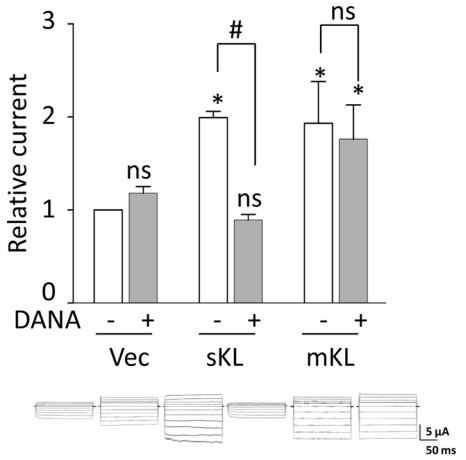

FIGURE 6. **The extracellular sialidase inhibitor DANA prevents TRPV5 up-regulation by secreted Klotho but not TRPV5 up-regulation by membranous Klotho.** Application of DANA to the cell culture medium had no effect on the TRPV5 whole-cell current of HEK cells cotransfected with the vector (*Vec*) control or mKL but completely abolished TRPV5 up-regulation by sKL. The *bar graph* shows mean ± S.E. ($n = 6$ for each) of TRPV5 current density at −150 mV. *, $p < 0.005$ compared with corresponding vector control-transfected HEK cells; #, $p < 0.001$; *ns*, not significant. Typical current traces are shown below each specific study group.

These results support the hypothesis that amino acids at positions corresponding to the nucleophile and acid-base catalyst glutamates of family 1 glycosidases are important for the putative sialidase activity of Klotho.

To investigate whether the intracellular action of Klotho toward TRPV5 mediated by membranous Klotho requires sialidase activity, we introduced the E416A, N417A, N690A, and E691A mutations to membranous Klotho and tested its effect to regulate TRPV5. As shown in Fig. 5*A*, cotransfection with wild-type but not any of the four membranous Klotho mutations increased TRPV5 current density, supporting the conclusion that the intracellular activity of Klotho toward TRPV5 involves the sialidase activity.

Lack of Effect of the Sialidase Inhibitor on the Regulation of TRPV5 by Membranous Klotho—We next tested the effect of a membrane-impermeant sialidase inhibitor, DANA, on the regulation of TRPV5 by secreted *versus* membranous Klotho. As reported previously, addition of DANA to the cell medium prevented the up-regulation of TRPV5 by the secreted Klotho (Fig. 6). For comparison, DANA failed to inhibit the up-regulation of TRPV5 by membranous Klotho. These results provide further support for the hypothesis that the effect of membranous Klotho on TRPV5 is due to its intracellular action and requires a sialidase activity.

Impaired Endocytosis Does Not Affect TRPV5 Up-regulation by Membranous Klotho—TRPV5 undergoes constitutive dynamin II-dependent endocytosis (30, 36). Secreted Klotho increases TRPV5 current activity and membrane abundance by modifying its *N*-glycan structure to decrease the removal of the channel from the cell surface by dynamin II-dependent endocytosis (30). Because up-regulation of TRPV5 by membranous Klotho also requires *N*-glycan, we examined the role of dynamin II-dependent endocytosis in the action of membranous Klotho. TRPV5 was cotransfected with wild-type or a dominant-negative dynamin II and with secreted or membranous Klotho. Klotho (sKL and mKL) increased TRPV5 current density in cells cotransfected with wild-type dynamin II (Fig. 7*A*) to a similar extent as in cells without cotransfection of dynamin II (Fig. 1*B*). This is likely due to the fact that HEK293 cells have abundant endogenous dynamin II.

TRPV5 undergoes constitutive dynamin II-dependent endocytosis. Therefore, cotransfection with dominant-negative dynamin II increased TRPV5 current density even in the absence of Klotho (Fig. 7*A*). Under the conditions in which constitutive endocytosis is impaired, secreted Klotho did not alter TRPV5 current density, supporting the notion that secreted Klotho increases TRPV5 channel activity by decreasing endocytosis. In contrast, membranous Klotho significantly increased the current density in this condition, indicating that its regulation of TRPV5 involves, at least partially, other additional mechanisms.

Brefeldin A Inhibits Up-regulation of TRPV5 by Membranous Klotho—*N*-glycosylation is also important for intracellular protein trafficking (32–34). We asked whether membranous Klotho might increase TRPV5 by enhancing forward trafficking of channel proteins using BFA, a fungal toxin known to inhibit the early secretory pathway by reversing ER-to-Golgi trafficking and, therefore, reversing intracellular forward trafficking (38). HEK cells cotransfected with TRPV5 and with secreted or membranous Klotho were treated with BFA or vehicle

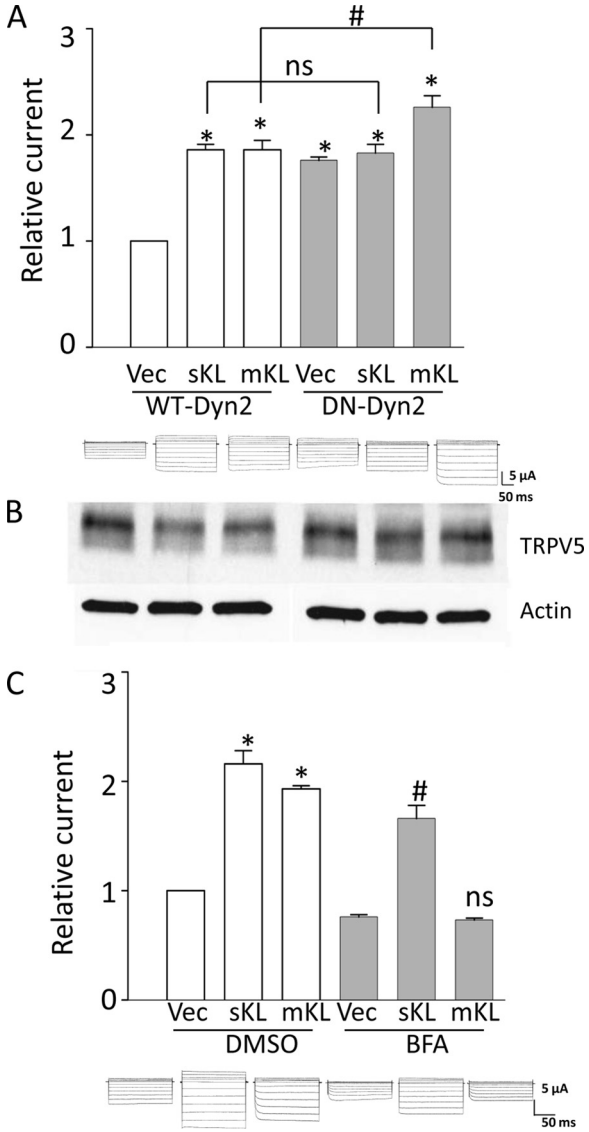

FIGURE 7. **Up-regulation of TRPV5 by membranous Klotho is not affected by impaired dynamin-II dependent endocytosis but is neutralized by impaired forward trafficking.** *A*, HEK cells were cotransfected with TRPV5, sKL, or mKL and with either wild-type (*WT-Dyn2*) or dominant-negative (K44A) dynamin II (*DN-Dyn2*). The *bar graph* shows mean ± S.E. ($n = 6$ for each) of TRPV5 current density at −150 mV. *, $p < 0.0005$ compared with vector + WT-Dyn2; #, $p < 0.001$; *ns*, not significant. Typical current traces are shown below each specific study group. *B*, TRPV5 protein density in total cell lysate is shown after cotransfection with control vector, wild-type sKL, or mKL with either WT-Dyn2 or DN-Dyn2. No significant difference regarding TRPV5 channel expression was detected between cells cotransfected with sKL or mKL. *C*, HEK cells were cotransfected with TRPV5 and with sKL or mKL. Cells were then treated with either dimethyl sulfoxide (*DMSO*, vehicle) or BFA (38). The *bar graph* shows mean ± S.E. ($n = 5$ for each) of TRPV5 current density at −150 mV. *, $p < 0.0005$ compared with dimethyl sulfoxide-treated, vector control-transfected HEK cells; #, $p < 0.001$ compared with BFA-treated vector (*Vec*) control; *ns*, not significant. Typical current traces are shown below each specific study group.

(dimethyl sulfoxide). Dimethyl sulfoxide had no effect on the up-regulation of TRPV5 by secreted or membranous Klotho (Fig. 7*C*). Treatment with BFA had no effect on the increase of TRPV5 current density by secreted Klotho, supporting the previous observation that secreted Klotho works by impairment of endocytosis. In contrast, BFA abolished the increase in TRPV5 current density by membranous Klotho. Treatment with BFA by itself had no deleterious effect on TRPV5 channel activity. Therefore, membranous Klotho increased TRPV5 cell surface abundance by enhancing BFA-inhibitable intracellular forward trafficking.

Up-regulation of TRPV5 by Klotho Is Independent of FGF23—mKL physically associates with FGFR to form the coreceptor for FGF23 (13–15). To exclude that the intracellular signaling cascade activated by FGF23 may underline the intracellular effect of mKL, we examined the effect of coexpression of sKL or mKL on TRPV5 in L6 cells that lack endogenous FGFRs (39). Using the same amount of Klotho plasmid DNA as in experiments performed in HEK cells (Figs. 1 and 6), both sKL and mKL up-regulated TRPV5 in L6 cells (Fig. 8, *A* and *B*), indicating that the intracellular effect of mKL is independent of FGF23 activation of the FGFR-mKL coreceptor. In these experiments, using L6 cells, there was a trend toward higher TRPV5 current density with mKL than with sKL (Fig. 8*A*). We performed further experiments using double the amount of Klotho plasmids in L6 cells and found that the magnitude of increase in TRPV5 current density was greater when cotransfected with mKL than with sKL (Fig. 8*C*). These results may be due to the possibility that transfection with a higher dose of mKL, although producing less secreted Klotho ectodomain in the medium compared with transfection with the same amount of sKL DNA, produces membranous Klotho that resides on the surface membrane in close proximity to TRPV5 so that it can regulate TRPV5 via the extracellular domain despite not being released in the medium. To support this notion of additional action of mKL tethered to the cell membrane, we found that extracellular application of DANA caused an apparent (although not statistically significant) decrease in TRPV5 currents in L6 cells transfected with a higher dose of mKL (Fig. 8*D*). In comparison, DANA completely abolished the increase of TRPV5 current density by coexpression with the same, higher amount of sKL.

DISCUSSION

Klotho is a type 1 transmembrane protein produced predominantly in the kidney. The extracellular domain of Klotho is shed and released into the systemic circulation and tissue fluids, including urine and CSF. The function of the secreted extracellular domain of Klotho remains largely unknown, but many studies have shown that it may act as a paracrine factor to regulate ion transport proteins such as the TRPV5 Ca^{2+} channel, the ROMK (renal outer medullary potassium channel) K^+ channel, and the renal sodium-phosphate cotransporter (NaP_i-2a) (18, 19, 30) or an endocrine factor to regulate growth factor signaling (20–22). With respect to TRPV5, secreted Klotho increases the cell surface abundance of TRPV5 via a putative sialidase activity to remove sialic acids from *N*-glycan of TRPV5 and enhance its binding to galectin-1, therefore decreasing its endocytosis and promoting retention at the cell surface (30).

In the kidney, Klotho is highly expressed in the renal distal tubules where TRPV5 channels coexpress. In this study, we investigated whether Klotho can regulate TRPV5 acting intracellularly. To demonstrate the intracellular action of Klotho in distinction from the extracellular action of secreted Klotho, we showed that the full-length membranous form of Klotho can up-regulate TRPV5 at a level of expression that does not result

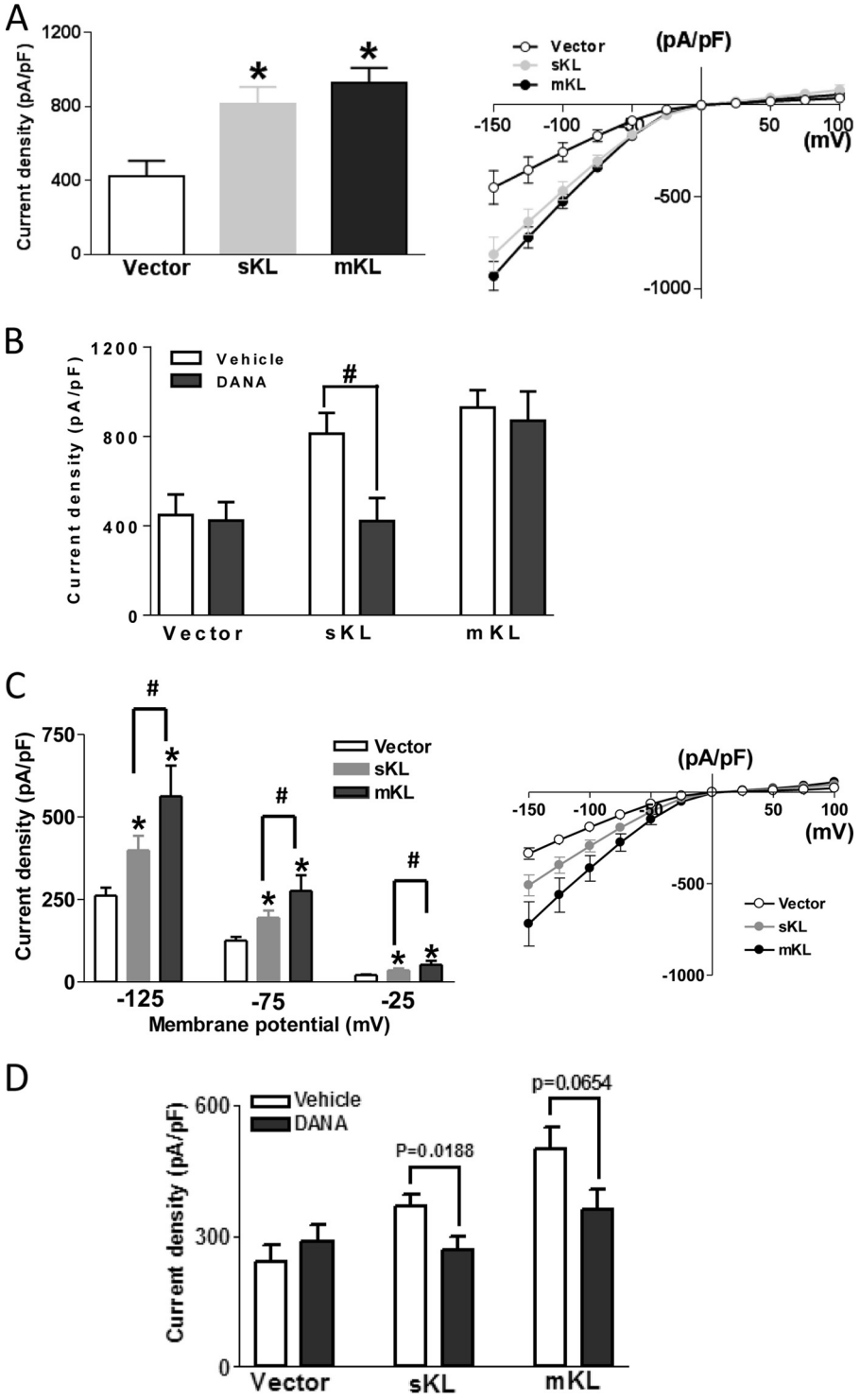

FIGURE 8. **The effect of Klotho on TRPV5 is independent of FGF23.** *A*, coexpression with cDNA coding for sKL or mKL (1000 ng each) both increased TRPV5 current density compared with vector in L6 cells lacking endogenous FGFRs. There was a trend toward higher TRPV5 current density with mKL, but it was not statistically significant. *B*, extracellular application of DANA completely abolished the increase of TRPV5 current density by coexpression with sKL but not with mKL. *C*, coexpression with cDNA coding for sKL or mKL (2000 ng each) both increased TRPV5 current density in L6 cells lacking endogenous FGFRs. The increase by mKL is greater than that by sKL. *D*, extracellular application of DANA completely abolished the increase of TRPV5 current density by coexpression with sKL (2000 ng cotransfected), and slightly (but not statistically significantly) diminished the increase by mKL (2000 ng cotransfected). The *bar graph* shows mean ± S.E. ($n = 6$ for each) of TRPV5 current density at indicated mV. *, $p < 0.01$ compared with vector; #, $p < 0.01$ between groups; *pF*, picofarad.

in a significant secretion into the cell culture medium. To exclude the possibility that the effect of membranous Klotho is caused by an undetectable level of secreted Klotho in the medium, we showed that a sialidase inhibitor, although completely preventing the effect of secreted Klotho, fails to inhibit the stimulation of TRPV5 by membranous Klotho. Together,

our results provide strong evidence to support the hypothesis that regulation of TRPV5 channels by Klotho may, in part, be due to an effect inside the cells.

Both extracellular and intracellular actions of Klotho require *N*-linked glycosylation of TRPV5 at asparagine 358. However, their mechanisms of action are different. In contrast to secreted Klotho, which modifies the mature *N*-glycan from the extracellular space to inhibit endocytosis of TRPV5, membranous Klotho appears to promote forward trafficking of channel proteins from the cell interior.

Previously, van de Graaf *et al.* (40) reported that BFA treatment did not alter TRPV5 activity, as analyzed by ruthenium red-inhibitable calcium uptake. In that study, they used an artificial TRPV5 construct that contains an additional transmembrane domain added to the amino terminus so that the construct encoded seven transmembrane domains and an extracellularly located N terminus as opposed to six transmembrane domains and an intracellularly localized N terminus in the native TRPV5 channel. Therefore, it may not be possible to compare our studies with their studies directly.

Recently, Andrukhova *et al.* (41) proposed that stimulation of membranous Klotho and the FGFR complex by FGF23 activates a signaling cascade involving ERK1/2, SGK1, and WNK4 to promote TRPV5 channel trafficking. The mechanism of action of membranous Klotho we observed here appears to be different from their proposed mechanism. First, we found that coexpression with mKL or sKL or the extracellular application of purified sKL activates TRPV5 in a cell line that lacks endogenous FGFR, indicating that our finding is not FGF23 ligand-dependent. Second, enzymatic activity appears to be important in our case. Conceivably, Klotho-mediated regulation of TRPV5 may involve multiple potential mechanisms. How Klotho regulates intracellular trafficking remains unclear. Interaction with galectins is known to enhance the intracellular trafficking of proteins (32, 42, 43). Klotho may desialylate *N*-glycan of TRPV5 within the Golgi to promote its binding to galectins and forward trafficking. One may wonder how the expression of recombinant sKL and mKL by transfection may result in different intracellular effects toward TRPV5 because both should be present within the ER-Golgi, although sKL would be in the lumen, whereas mKL would be in the ER-Golgi membrane. We suggest that a closer proximity of mKL and TRPV5 in the ER-Golgi membrane would facilitate the enzymatic modification of TRPV5 by mKL via the putative sialidase activity. In the native tissue, secreted Klotho is probably not very abundant, if at all present, in the lumen of the ER-Golgi because secreted Klotho originates predominantly from cleavage of cell-surface membranous Klotho by metalloproteases (8). Alternatively, intracellular Klotho may work indirectly through other process(es) distinct between mKL and sKL. Future studies will investigate these questions.

Activation of the membranous Klotho-FGFR complex suppresses 1,25-dihydroxyvitamin D synthesis to decrease phosphate and calcium absorption from the gut (17, 18). The stimulation of TRPV5 by Klotho will counter the effect of suppressed vitamin D synthesis on the calcium balance to allow the FGF23-Klotho/FGFR axis to regulate the body phosphate balance independently of the calcium balance.

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