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ION CHANNELS, RECEPTORS AND TRANSPORTERS

Casein kinase-mediated phosphorylation of serine 839 is necessary for basolateral localization of the Ca^{2+}-activated non-selective cation channel TRPM4

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Abstract Transient receptor potential melastatin-like 4 (TRPM4) is a Ca^{2+}-activated non-selective cation channel expressed in a wide range of human tissues. TRPM4 participates in a variety of physiological processes such as T cell activation, myogenic vasoconstriction, and allergic reactions. TRPM4 Ca^{2+} sensitivity is enhanced by calmodulin (CaM) and phosphathydilinositol 4, 5-bisphosphate ($PI(4,5)P_2$) binding, as well as, under certain conditions, PKC activation. However, information as to the mechanisms of modulation of this channel remains unknown, including direct identification of phosphorylation sites on TRPM4 and their role in channel features. Here, we use mass-spectrometric-based proteomic approaches (immunoprecipitation and tandem mass spectrometry) to unambiguously identify S839 as a phosphorylation site present on human TRPM4 expressed in a human cell line. Site-directed mutagenesis employing a serine to alanine mutation to eliminate phosphorylation, and a phospho-mimetic aspartate mutation, as well as biochemical and immunocytochemical experiments, revealed a role for S839 phosphorylation in the basolateral expression of TRPM4 channels in epithelial cells. Moreover, we demonstrated that casein kinase 1 (CK1) phosphorylates S839 and is responsible for the basolateral localization of TRPM4.

Keywords TRP channels · LC-MS/MS · Basolateral · Phosphorylation · Casein kinase

Abbreviations

TRPM4	Transient receptor potential melastatin-like 4
$PI(4,5)P_2$	Phosphatidylinositide 4, 5-bisphosphate
PKC	Protein kinase C
LC-MS/MS	Liquid chromatography coupled-tandem mass spectrometry

Introduction

Transient receptor potential melastatin-like 4 (TRPM4) is a Ca^{2+}-activated non-selective cation channel (Ca^{2+}-NSCC), expressed in a wide range of human tissues, where it regulates a variety of physiological and pathophysiological processes, including cellular death and proliferation, insulin secretion, T-cell differentiation, and dendritic and mast cell migration [1, 2, 6, 8, 13–16, 20, 22]. Although TRPM4 is intrinsically Ca^{2+} impermeant, in non-excitable cells that lack voltage-gated Ca^{2+} channels, it plays an important effect in regulating intracellular Ca^{2+} oscillations by providing membrane

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depolarization necessary for Ca^{2+} influx via store operated channels (SOCs) [23]. Physiologically, it has been shown that TRPM4 knockdown alters Ca^{2+} oscillations during T-cell activation, leading to a failure in interleukin-2 secretion [23], and TRPM4 knockout mice exhibit a notable loss in dendritic cell migration and diminished mast cell IgE secretion [2, 49]. TRPM4 has also been linked to cerebral blood flow and insulin secretion from pancreatic β-cells [6, 14, 26, 30, 35]. Pathophysiological roles of TRPM4 have also been recently elucidated. A direct role of this channel has been shown in hypertension and cardiac diseases [20, 25, 24, 43], and its participation in LPS and H_2O_2-induced cell death has also been described [3, 41].

In spite of the important role of TRPM4 in a variety of cellular processes, little is known about the physiological regulation of this channel. Previous studies have shown that binding of calmodulin (CaM) and $PI(4,5)P_2$ enhance the Ca^{2+} sensitivity of TRPM4 [32, 54]. Phorbol ester treatment led to enhanced Ca^{2+} sensitivity of recombinant human TRPM4 expressed in human HEK293 cells. This treatment affected wild-type TRPM4 but not of two mutants (S1145A or S1152A) at putative PKC phosphorylation sites located on the TRPM4 cytoplasmic C-terminus [31]. In addition, phorbol ester treatment also affected trafficking and surface expression of GFP-tagged mouse TRPM4 expressed in smooth muscle cells [12]. Phosphorylation is a major mechanism in cellular homeostasis and the most common post-translational modification [47], and human TRPM4 contains ~130 serine and threonine and ~27 tyrosine residues in its sequence that can be potentially phosphorylated by different kinases. Here, we performed a mass spectrometric-based proteomics analysis to identify the sites that chemically modified with phosphate on immunopurified human TRPM4 channels. Using liquid chromatography-tandem mass spectrometry (LC-MS/MS), we unambiguously identified S839 as a novel phosphorylation site on the human TRPM4 protein. We provide the first immunochemical evidence for the basolateral localization of the TRPM4 channels in polarized epithelial cells, and that phosphorylation at S839 is crucial for TRPM4 basolateral localization via casein kinase 1 (CK1)-dependent phosphorylation.

Materials and methods

Cell culture and plasmids

HEK293 cells were cultured in DMEM low glucose/F12 medium (1:1), supplemented with 5 % fetal bovine serum. TREx-TRPM4 cells were generated by stable transfection of the pcDNA4TO/hTRPM4 plasmid (kindly provided by Dr P. Launay) into TREx293 cells (Invitrogen, Carlsbad, CA) using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA) followed by selection with 5 μg/mL blasticidin. These cells were subsequently grown in the same medium used for HEK293 cells. A single positive clone (clone 11) was selected for phosphorylation residue identification using LC-MS/MS. MDCK cells were cultured in DMEM high glucose medium supplemented with 10 % fetal bovine serum. Twenty-four hours before transfection, cells were grown in Ca^{2+}-free medium and then were plated to 50 % confluence on Transwell Permeable Support (0.4 μm polycarbonate membrane; Costar, Corning, NY) and transfected 12 h later with 500 ng plasmid DNA/well. Cells were transferred to Ca^{2+}-containing medium and grown for an additional 4 days after transfection to establish a confluent monolayer. All cell lines were grown at 37 °C and 5 % CO_2.

Antibodies

For immunofluorescence, immunoblot and immunoprecipitation experiments, we used the following antibodies: rabbit polyclonal (pAb) anti-FLAG antibody (Sigma-Aldrich, St Louis, MO), rabbit pAb anti-phosphoserine (pS) (ab9332, Abcam), mouse monoclonal (mAb) IgG1 anti-Grp75 (N52A/42, UC Davis/NIH NeuroMab Facility, Davis, CA), mouse mAb IgG1 anti-E-cadherin (RR1, DSHB, Iowa City, IA), and mouse mAb IgG2b 12CA5 anti-HA (BACO, Berkeley, CA).

Immunoprecipitation and immunoblotting

TRPM4 protein expression in TREx-TRPM4 cells was induced by adding 1 μg/mL tetracycline to the medium and incubating for 24 h. The cells were then lysed in lysis buffer containing 1 % *v/v* Triton X-100, 150 mM NaCl, 1 mM EDTA, 50 mM Tris–HCl (pH 7.4), 1 mM sodium orthovanadate, 5 mM NaF, 1 mM PMSF, aprotinin (1.5 μg/mL), antipain (10 μg/mL), leupeptin (10 μg/mL), and benzamidine (100 μg/mL). The lysates were centrifuged at 12,000*g* at 4 °C for 10 min. The supernatants were incubated with anti-FLAG column (Sigma-Aldrich, St Louis, MO) overnight at 4 °C. After incubation, immunoprecipitation reaction products were washed six times in wash buffer (150 mM NaCl, 1 mM EDTA, 50 mM Tris–HCl, pH 7.4) and immunocomplexes were eluted with reducing sample buffer (62.5 mM Tris–HCl, 2 % *w/v* SDS, 10 % *v/v* glycerol, 1 % *v/v* β-mercaptoethanol) and size-fractionated on 6–12 % gradient SDS–PAGE, according to the Laemmli method [21].

In gel digestion

The TRPM4 band (~140 kDa) was directly excised from Colloidal Coomassie blue stained gels [33], destained with 50 % *v/v* acetonitrile in 25 mM ammonium bicarbonate, and dried in a speed vacuum concentrator. Dried gel pieces were reswollen with 25 mM ammonium bicarbonate (pH 8.0)

containing 50 ng/μL trypsin (Promega, Valencia, CA) and incubated at 37 °C for 16–24 h. Supernatant peptide mixtures were extracted with 50 % *v/v* acetonitrile in 5 % *v/v* formic acid and dried in a speed vacuum concentrator.

Mass spectrometry analysis

An HPLC system (Paradigm MG4, Michrom Bio Resources, Auburn, CA) directly coupled with an ion trap mass spectrometer (LTQ-FT, Finnigan, San Jose, CA) was used for microcapillary LC-MS/MS data acquisition. A homemade fritless reverse phase (RP) microcapillary column (0.1× 180 mm) was packed with C18 (Phenomenex, Aqua: 5 μm 300 Å). Tryptic peptide mixtures were desalted and concentrated using the RP trap column (0.15×30 mm) before chromatographic separation by the microcapillary RP column with a flow rate of 300 nL/min. Eluted peptides were then directly sprayed into the mass spectrometer. MS/MS spectra were acquired for the most intense peptide ion from the previous MS spectra with dynamic exclusion for 3 min. The two buffers used for the RP chromatography were 5 % *v/v* acetonitrile in 0.1 % *v/v* formic acid (buffer A) and 80 % *v/v* acetonitrile in 0.1 % *v/v* formic acid (buffer B). A 2.5-h long gradient (0–10 % B for 10 min, 10–45 % B for 110 min, 45–100 % B for 20 min, and 100 % B for 10 min) was used for the maximum separation of the peptides. Mascot searches for LC-MS/MS data were performed for the MS/MS data set against the human TRPM4 protein sequence with differential phosphorylation on Tyr, Thr, and Ser and oxidation on Met. Peptide sequences from the search result were filtered out using DTASelect software (*5*) with a slightly lower criteria than typically used for protein identification as to not miss any possible phosphopeptides: correlation value (Xcorr)>1.5, 2.0, and 3.0 for singly, doubly, and triply charged tryptic peptide ions, respectively, and ΔCn (the difference in correlation with the next highest Xcorr) higher than 0.08. Each filtered MS/MS spectra exhibiting possible phosphorylation was manually checked and validated. Existence of a 98 dalton mass loss ($-H_2PO_4$: phosphopeptide-specific CID neutral loss) and any ambiguity of phosphorylation sites were carefully examined.

Site-directed mutagenesis and transient transfection

Mutagenesis of recombinant human TRPM4 cDNA in the pcDNA4TO vector was performed using the Quick-change site-directed mutagenesis kit (Stratagene, La Jolla, CA) as per the manufacturer's instructions. All mutants were checked by DNA sequencing. HEK293 cells were plated in 35 mm plastic tissue culture dishes and transfected with wild-type or mutant pcDNA4TO/hTRPM4 plasmid (0.1 μg) using Lipofectamine 2000 reagent (Invitrogen). All experiments were performed 48 h post transfection.

Immunofluorescence

For labeling of the apical membrane surface, MDCK cells were incubated for 30 min at 4 °C with 20 μg/mL FITC-WGA in PBS (10 mM phosphate buffer, pH 7.4, 150 mM NaCl, containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$). Cells were washed three times and then were fixed for 15 min at room temperature in fixing solution, permeabilized and incubated in blocking solution for 45 min at room temperature, and inmunostained with anti-FLAG and mouse monoclonal anti-E-cadherin diluted in blocking solution. Primary antibodies were detected with Alexa 488 goat anti-mouse IgG1 and Alexa 594 goat anti-rabbit or with Alexa 594 goat anti-mouse IgG1 and Alexa 350 goat anti-rabbit secondary antibodies (Invitrogen, Carlsbad, CA), and nuclei were stained with Hoechst 33258 (200 ng/mL). Images were acquired with a CCD camera installed on Axiovert 200 M microscope (Zeiss, Oberkochen, Germany) with ×100, 1.3 numerical aperture objectives, and an ApoTome coupled to Axiovision software (Zeiss, Oberkochen, Germany).

Surface biotinylation

HEK293 cells were grown to 80–90 % confluence on poly-L-lysine (200 μg/mL) treated 35 mm tissue culture dishes. Cells were then washed twice with ice-cold DPBS (pH 8.0) and incubated with 500 μg/mL EZ-link sulfo-NHS-LC-biotin (Thermo, Rockford, IL) dissolved in DPBS (pH 8.0) for 30 min at room temperature. The reaction was terminated with blocking solution (50 mM Tris–HCl, 154 mM NaCl, pH 8.0), and the cells were washed twice with ice-cold PBS. Cells labeled with sulfo-NHS-LC-biotin were lysed as described above, and lysates were incubated with NeutrAvidin Agarose (Thermo, Rockford, IL) overnight at 4 °C. The precipitated proteins were eluted with reducing sample buffer and resolved in SDS-PAGE for further immunoblot analysis.

Results

Identification of a novel TRPM4 phosphorylation site

To identify phosphorylation sites on TRPM4, we used a previously characterized stable cell line expressing a FLAG-tagged human TRPM4 under the control of a tetracycline-inducible operator (TREx-TRPM4) [41]. Stimulation of these cells with 1 μg/mL tetracycline for 16 h induces high levels of TRPM4 protein expression (Fig. 1a), in contrast to non-stimulated cells, in which no expression of TRPM4 was detected. In order to identify sites chemically modified with phosphate, we purified the recombinant human TRPM4 protein from induced TREx-TRPM4 cells by immunoaffinity chromatography using an anti-FLAG antibody. The purified

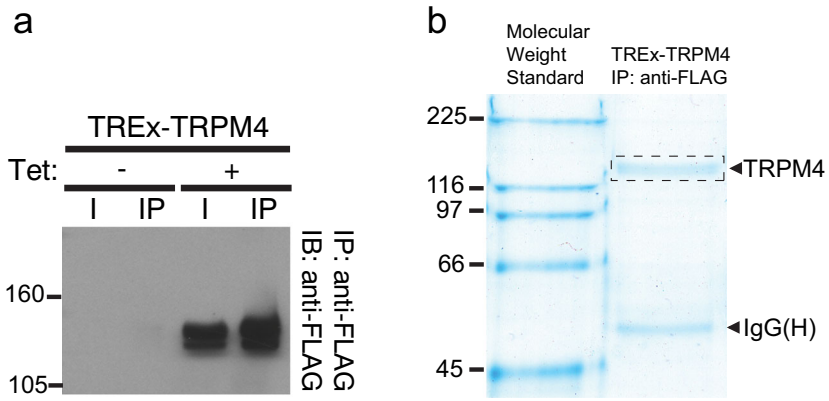

Fig. 1 Immunopurification of hTRPM4 from a plasma membrane-enriched protein fraction from tetracycline-induced (+) and non-induced (−) TREx-TRPM4 cells. **a** SDS-PAGE separation of eluates from immunoprecipitation of TRPM4 with anti-FLAG antibody. Immunoblot of input (I) and immunoprecipitation products (IP) of TRPM4 from TREx-TRPM4 cells after SDS-PAGE separation. **b** Coomassie brilliant blue stained SDS-PAGE of a large-scale immunopurification showing the yield of the FLAG-TRPM4 channels obtained with the anti-FLAG immunopurification from tetracycline-induced TREx-TRPM4 cells. The *left lane* corresponds to the molecular weight standards in kDa

protein was then size fractionated by SDS-PAGE (Fig. 1b), and the Colloidal Coomassie blue stained TRPM4 band excised. Following *in gel* trypsin digestion, we analyzed these samples by LC-MS/MS. The TRPM4 peptides were identified using a Mascot and Transproteomic Pipeline database search, resulting in 349 independent spectra that matched human TRPM4, resulting in 63.8 % overall coverage of the TRPM4 protein, and 70.7 % coverage of the cytoplasmic regions (76.5 % of the 684 a.a. of the cytoplasmic N-terminal segment and 69.0 % of the 174 a.a. C-terminal segment). Using this approach, we identified phosphorylated residue pS839, located on a tryptic peptide (^{831}QGLSGGGGpSLASGGPGPGH ASLSQR^{855}) derived from the S2-S3 cytoplasmic linker of TRPM4 (Fig. 2a–b).

Phosphorylation at S839 does not regulate cell surface expression of TRPM4 channels

To determine the possible role of S839 phosphorylation on the expression of TRPM4 at the plasma membrane, we transiently transfected HEK293 cells with wild-type TRPM4, the phosphorylation disrupting mutant TRPM4 S839A, and the phosphomimetic mutant TRPM4 S839D, and performed cell surface biotinylation labeling assays. In these experiments, we observed that both mutant TRPM4 proteins (S839A and S839D) are expressed in the plasma membrane at a level similar to wild-type TRPM4 protein (Fig. 3). Therefore, we concluded that the phosphorylation on S839 is not related to a diminished expression on the plasma membrane.

Phosphorylation at S839 regulates basolateral localization of TRPM4 channels

TRPM4 channels are expressed in a polarized manner in kidney [10, 17] and bladder [53] epithelial cells. To determine the role of phosphorylation at S839 in polarized expression, we transiently transfected wild-type TRPM4 and the phosphosite mutants (S839A and S839D) in polarized canine kidney epithelial MDCK cells. We then performed immunofluorescence staining and optical sectioning microscopy on polarized confluent MDCK cell monolayers to determine the localization of the exogenous channel proteins. We labeled the apical domain with FITC-WGA before cell permeabilization and immunostaining, and counterstained cells for E-cadherin as a basolateral marker. In these experiments, we observed that wild-type TRPM4 channels exhibited clear basolateral localization in every transfected cell, as showed by its colocalization with E-cadherin (Fig. 4a) and lack of colocalization with WGA (Fig. 4b). In a minor population of cells (15.0±3.9 %), apical localization of wild-type TRPM4 was also observed, as demonstrated by clear colocalization with the WGA labeling (Fig. 4). This suggests that polarized expression of TRPM4 in epithelial cells has a component that is variable and/or conditional. Interestingly, the phosphomimetic TRPM4 S839D mutant showed similar basolateral pattern to wild-type TRPM4 (Fig. 4). Conversely, the phosphorylation disrupting TRPM4 S839A mutant showed an apical localization, as shown by colocalization with WGA, in the vast majority (74.1±1.9 %) of the cells analyzed. The TRPM4 S839A mutant was also found in the basolateral domain of the same cells (Fig. 4). We conclude that phosphorylation of the S839 residue controls the polarized expression of TRPM4 channels in epithelial cells, but not the biosynthetic trafficking of TRPM4 to the cell surface.

Casein kinase 1 (CK1) regulates basolateral localization of TRPM4 channels

In order to identify the kinase responsible for the phosphorylation of the S839 residue, we performed bioinformatics

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1     MVVPEKEQSW IPKIFKKKTC TTFIVDSTDP GGTLCQCGRP RTAHPAVAME
51    DAFGAAVVTV WDSDAHTTEK PTDAYGELDF TGAGRKHSNF LRLSDRTDPA
101   AVYSLVTRTW GFRAPNLVVS VLGGSGGPVL QTWLQDLLRR GLVRAAQSTG
151   AWIVTGGLHT GIGRHVGVAV RDHQMASTGG TKVVAMGVAP WGVVRNRDTL
201   INPKGSFPAR YRWRGDPEDG VQFPLDYNYS AFFLVDDGTH GCLGGENRFR
251   LRLESYISQQ KTGVGGTGID IPVLLLLIDG DEKMLTRIEN ATQAQLPCLL
301   VAGSGGAADC LAETLEDTLA PGSGGARQGE ARDRIRRFFP KGDLEVLQAQ
351   VERIMTRKEL LTVYSSEDGS EEFETIVLKA LVKACGSSEA SAYLDELRLA
401   VAWNRVDIAQ SELFRGDIQW RSFHLEASLM DALLNDRPEF VRLLISHGLS
451   LGHFLTPMRL AQLYSAAPSN SLIRNLLDQA SHSAGTKAPA LKGGAAELRP
501   PDVGHVLRML LGKMCAPRYP SGGAWDPHPG QGFGESMYLL SDKATSPLSL
551   DAGLGQAPWS DLLLWALLLN RAQMAMYFWE MGSNAVSSAL GACLLLRVMA
601   RLEPDAEEAA RRKDLAFKFE GMGVDLFGEC YRSSEVRAAR LLLRRCPLWG
651   DATCLQLAMQ ADARAFFAQD GVQSLLTQKW WGDMASTTPI WALVLAFFCP
701   PLIYTRLITF RKSEEEPTRE ELEFDMDSVI NGEGPVGTAD PAEKTPLGVP
751   RQSGRPGCCG GRCGGRRCLR RWFHFWGAPV TIFMGNVVSY LLFLLLFSRV
801   LLVDFQPAPP GSLELLLYFW AFTLLCEELR QGLSGGGGSL ASGGPGPGHA
851   SLSQRLRLYL ADSWNQCDLV ALTCFLLGVG CRLTPGLYHL GRTVLCIDFM
901   VFTVRLLHIF TVNKQLGPKI VIVSKMMKDV FFFLFFLGVW LVAYGVATEG
951   LLRPRDSDFP SILRRVFYRP YLQIFGQIPQ EDMDVALMEH SNCSSEPGFW
1001  AHPPGAQAGT CVSQYANWLV VLLLVIFLLV ANILLVNLLI AMFSYTFGKV
1051  QGNSDLYWKA QRYRLIREFH SRPALAPPFI VISHLRLLLR QLCRRPRSPQ
1101  PSSPALEHFR VYLSKEAERK LLTWESVHKE NFLLARDKK RESDSERLKR
1151  TSQKVDLALK QLGHIREYEQ RLKVLEREVQ QCSRVLGWVA EALSRSALLP
1201  PGGPPPPDLP GSKD
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b

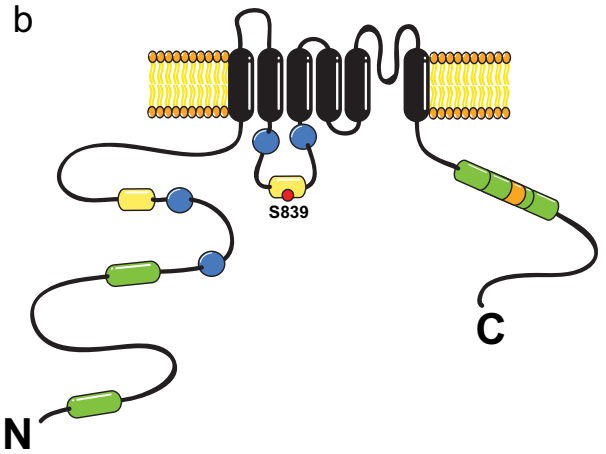

Fig. 2 Identification of phosphosites on recombinant hTRPM4 expressed in HEK293 cells using LC-MS/MS. **a** Deduced amino acid sequence of human TRPM4. The single phosphoresidue identified by LC-MS/MS is in *red*. The sequence coverage is indicated in *bold*. *Underlined regions* correspond to the predicted transmembrane segments of the channel. **b** *Cartoon* indicating the localization of the identified phosphosite at S839. CaM binding motifs (*green*), ABC transporter motifs (*yellow*), Walker B motifs (*blue*) and PH domain (*orange*) are shown. N and C denote amino- and carboxyl-terminal regions

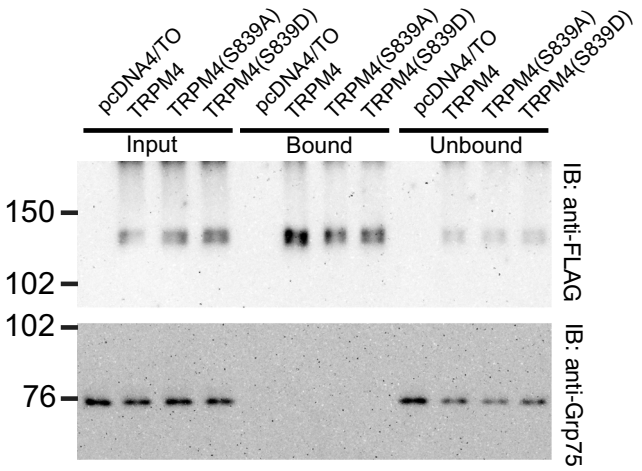

Fig. 3 Loss of phosphorylation in the S839 residue does not affect TRPM4 expression in the plasma membrane. HEK293 cells were transiently transfected with TRPM4, TRPM4 S839A or TRPM4 S839D, and surface-labeled with 500 μg/mL EZ-link sulfo-NHS-LC-biotin. The biotinylated proteins purified by NeutrAvidin Agarose, eluted with 1X RSB, resolved in SDS-PAGE, and analyzed by immunoblot using anti-FLAG antibody for TRPM4 detection. The immunoblot for the intracellular protein Grp75 was included as cytosolic control

analyses of the S2-S3 loop sequence, comparing it with the Human Protein Reference database (www.hprd.org). Interestingly, we found that the sequence residues S839–S842 (^{839}SLAS^{842}) and containing pS839 constitute a putative non-canonical consensus motif for CK1 phosphorylation [27], a highly conserved ubiquitous S/T kinase. Therefore, we hypothesized that this protein kinase might be involved in TRPM4 phosphorylation. In order to explore this possibility, we coexpressed wild-type TRPM4 with wild-type CK1ε (CK1-WT) and a kinase-dead (K38R) dominant-negative version of CK1ε (CK1-DN). Then, we immunopurified FLAG-TRPM4 with an anti-FLAG antibody, and serine residues phosphorylation were determined by immunoblot using a phosphoserine antibody (pS). We observed an increase in the immunoreactivity for the pS antibody against TRPM4 present in the CK1 cotransfected cells (Fig. 5a). Conversely, we observed diminished TRPM4 phosphorylation in the presence of the CK1-DN construct, similar to the loss of phosphorylation observed in the S839A mutant (Fig. 5a). Together, these data demonstrate a critical role for CK1 in TRPM4 phosphorylation.

We next determined whether CK1 activity is required for TRPM4 basolateral localization. We cotransfected MDCK cells with wild-type TRPM4 and CK1-DN, and then performed immunofluorescence staining and optical sectioning microscopy on polarized confluent MDCK cell monolayers. In these experiments, we observed that CK1-DN coexpression disrupts the basolateral localization of wild-type TRPM4, yielding a similar localization to the S839A mutant (Fig. 5b, d). This result suggests that CK1 regulates TRPM4 localization to the basolateral membrane. Because the S839 residue is a putative CK1 substrate, we hypothesized that this residue is required for the CK1-dependent localization. Therefore, we predicted that the TRPM4 S839D phosphomimetic mutant would be resistant to the effect of the CK1-DN. As shown in Fig. 5b, we observed that, unlike wild-type TRPM4, the S839D mutant still localizes to the basolateral membrane in cells coexpressing CK1-DN. In line with this finding, we hypothesized that coexpression of CK1-WT would not rescue the loss of localization of the S839A mutant. We then cotransfected CK1-WT with the S839A mutant and found that CK1-WT coexpression does not rescue the loss of basolateral localization of this mutant (Fig. 5c, e). In summary, these data suggest that CK1 regulates the basolateral localization of TRPM4 through phosphorylation of TRPM4 at S839.

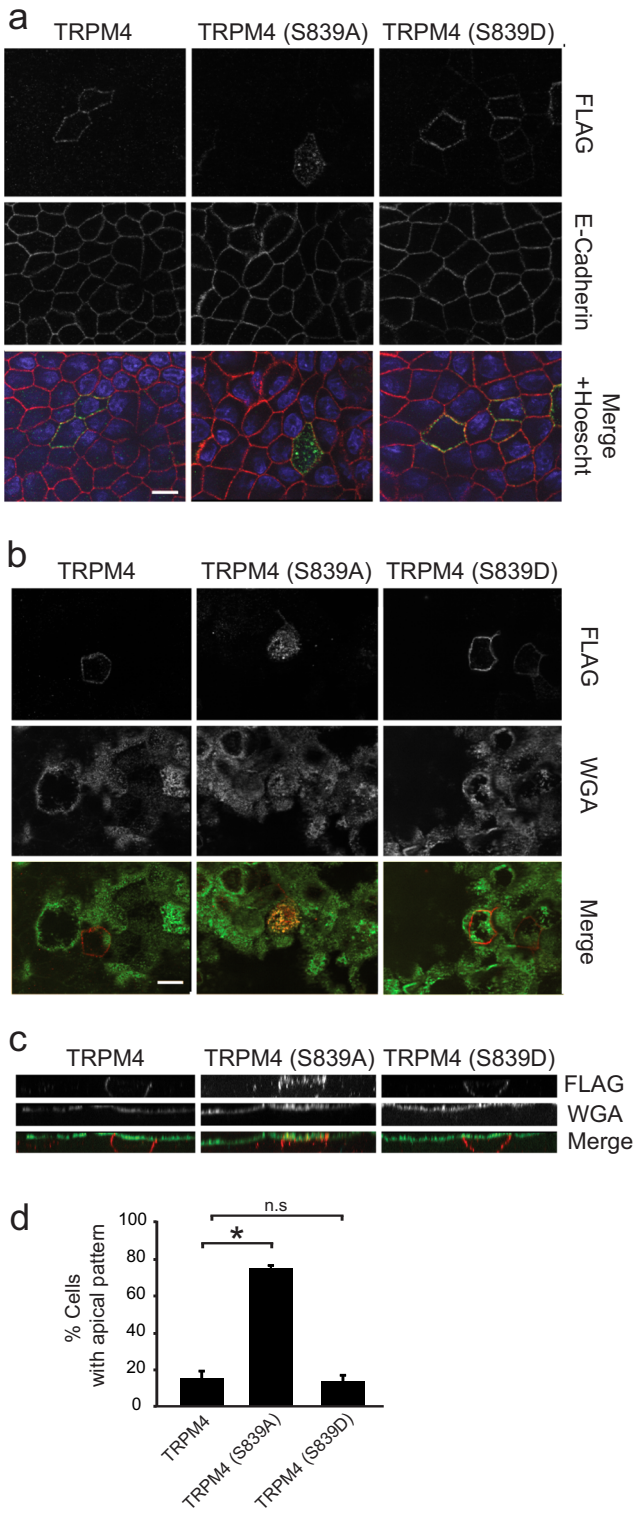

Fig. 4 Phosphorylation at the S839 residue is required for proper TRPM4 basolateral localization. MDCK cells were grown as confluent polarized monolayers after transfection with TRPM4, TRPM4 S839A, or TRPM4 S839D. The apical domain was labeled prior to fixation with WGA. After fixation, cells were immunostained with pAb anti-FLAG (*green*) and mAb anti-E-cadherin. Nuclei were stained with Hoechst (*blue*). Scale bar, 10 μm. **a** Monolayer of MDCK cells focused at a section midway through the cells, the basolateral domain is labeled for E-cadherin (*red*). **b** Monolayer of transfected MDCK cells focused at the apical domain, as labeled with WGA (*red*). **c** Lateral view of monolayer showing the differences in localization between the different versions of TRPM4. **d** Graph shows data on percentage of cells which exhibited apical localization of TRPM4 isoforms. *Corresponds to significant differences (ANOVA test, post-Dunnett, $p<0.01$) versus wild-type TRPM4. Data are from three independent experiments including $n=$ 559, 498, and 417 cells for TRPM4, TRPM4 S839A, and TRPM4 S839D, respectively

Discussion

Phosphorylation modulates numerous aspects of ion channel activity, such as voltage-dependence of activation, ligand binding and subcellular trafficking, and subcellular localization [7]. Proteomic approaches have been used to identify phosphorylation sites in several members of different families of ion channels, such as Nav, Kv, and TRP channels [5, 19, 33, 34, 38, 45, 51, 52]. Here, we used mass spectrometric-based proteomic approaches to identify a novel phosphorylated serine residue, S839, on recombinant human TRPM4 channel protein immunopurified from the human kidney HEK293 cell line. A previous study using HEK293 cells expressing recombinant human TRPM4 showed that phorbol ester stimulation altered the Ca^{2+} sensitivity of TRPM4 channel activation [31]. More recently, high-throughput proteomics studies revealed phosphorylation of mouse TRPM4 at T524, S527, S538, and Y577 [9, 18, 36, 37]. We did not identify phosphorylation on analogous residues in human TRPM4, suggesting species- or tissue-specific differences in TRPM4 phosphorylation. We also did not find evidence for phosphorylation at the sites suggested to mediate the phorbol ester response, perhaps suggesting a requirement for phorbol stimulation [31]. Whether the lack of detectable phosphorylation at these sites in our study is due to differences in sample preparation and/or analysis, or to biological differences (e.g., highly dynamic and and/or tissue-specific phosphorylation at these sites) is not known.

The S839 phosphorylation site is located in the S2-S3 linker (Fig. 2b) in the second ABC signature motif of TRPM4 (^{833}LSGGGGpSLASGGP^{845}). Interestingly, this motif also contains the G844 residue that is mutated with aspartate (i.e., G844D) as the major mutation leading to increased TRPM4 currents in cardiac conduction disease [24]. That the G844D mutation results in a neutral (G) to negatively charged (D) amino acid substitution suggest that alterations in the electrostatic environment of that region is important for determining the level of activity of TRPM4. That phosphorylation at S839, which would also contribute negative charges to this region, impacts the polarized localization of TRPM4 in epithelial cells shows the multifaceted nature of regulation mediated by the S2-S3 linker domain.

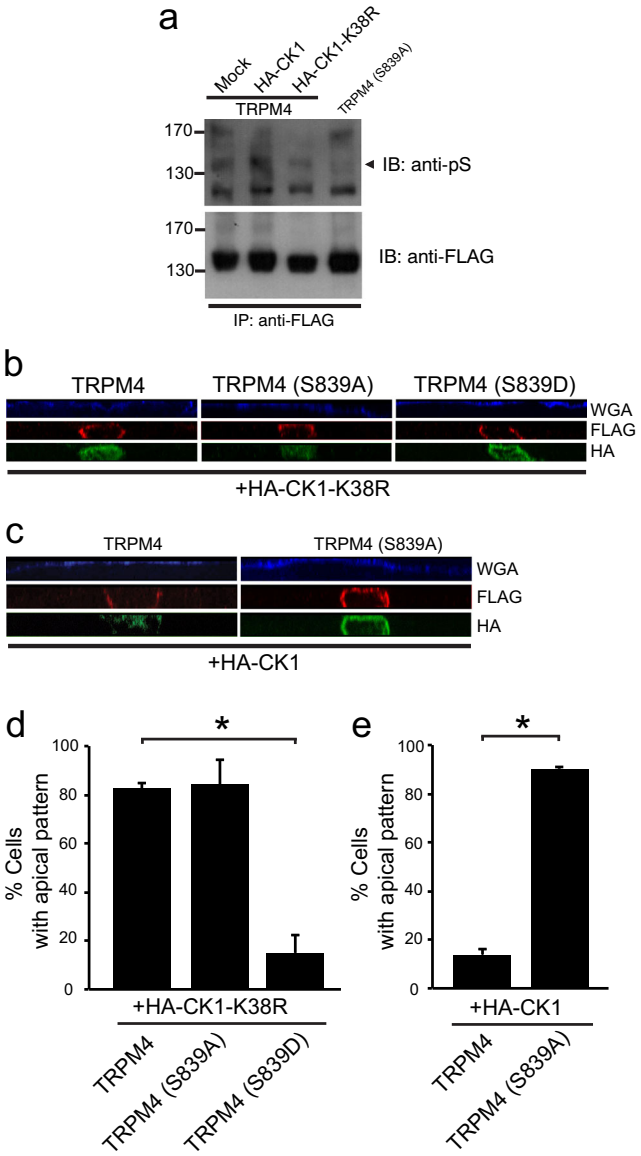

Fig. 5 CK1 activity is required for TRPM4 phosphorylation and proper basolateral localization. **a** HEK293 cells were transiently transfected with wild-type TRPM4 and HA-tagged CK1-DN or CK1, and TRPM4 S839A. FLAG-TRPM4 channels were immunopurified with an anti-FLAG antibody, and phosphorylation of Ser residues was determined by immunoblot using a phosphoserine antibody (anti-pS). *Arrowhead* indicates the phosphorylated form of TRPM4. **b**–**e** MDCK cells were grown as confluent polarized monolayers after cotransfection with TRPM4, TRPM4 S839A or TRPM4 S839D and either HA-tagged CK1-DN or CK1. The apical domain was stained prior to fixation with WGA (*blue*). After fixation, cells were immunostained with pAb anti-FLAG (*red*) and mAb anti-HA (*green*). **b** Lateral view of monolayer showing the localization of the different versions of TRPM4 cotransfected with HA-CK1-DN (HA-CK1-K38R). **c** Lateral view of MDCK cells monolayer showing the differences of localization between TRPM4 and TRPM4 S839A cotransfected with HA-CK1. **d** Graph shows data on percentage of cells which exhibited apical localization of TRPM4 isoforms cotransfected with HA-CK1-DN. *Corresponds to significant differences (ANOVA test, post-Dunnett, $p<0.01$) versus wild-type TRPM4. Data are from two independent experiments including $n=68$, 46, and 45 cells for TRPM4, TRPM4 S839A, and TRPM4 S839D, respectively. **e** Graph shows data on percentage of cells which exhibited apical localization of TRPM4 isoforms cotransfected with HA-CK1. *Corresponds to significant differences (student t test, $p<0.01$) versus wild-type TRPM4. Data are from two independent experiments including $n=43$ and 72 cells for TRPM4 and TRPM4 S839A, respectively

Epithelial tissue is a highly organized structure. Mechanisms for controlling the polarized expression of basolateral and apical protein populations represent a key process for determining the epithelial function and homeostasis. Therefore, the rupture of the epithelial polarity due to the altered subcellular localization of these protein populations can cause diverse pathologies, such as chronic inflammation and cancer [42]. Previously, the presence of TRPM4-like currents in the basolateral domain of mouse renal tubules has been demonstrated [10, 17]. Moreover, there are several reports regarding basolateral localization of nonselective calcium-activated cationic channels in different epithelia [39, 46, 48]. Here, we provide the first immunocytochemical evidence that demonstrates the specific basolateral localization of TRPM4 channels in polarized epithelial cells. By being located at the basolateral membrane, these channels may contribute to renal homeostasis. Since MDCK cells most probably correspond to renal absorptive tubular epithelium, a basolateral localization of TRPM4 may, upon channel activation, provide increased cation flux that initially stimulates the Na^+/K^+ pump, thereby triggering apical Na^+ reabsorption.

Posttranslational modifications of proteins, such as phosphorylation, constitute a major mechanism in the overall regulation of protein function, activity, trafficking, and localization [7]. It is no surprise that the activity and localization of many different ion channels are greatly influenced by phosphorylation-dependent mechanisms [7]. The Src-family protein tyrosine kinase (SFK) increases basolateral K^+ channels activity via angiotensin II stimulation of SFK activity in the thick ascending limb of the rat kidney [50], and SFK stimulates basolateral K^+ channel activity in mouse initial distal convoluted tubule by phosphorylating the Y9 residue on KCNJ10 channel [55]. Conversely, PKA-dependent phosphorylation of the S256 residue of aquaporin 2 (AQP2) is critical for its apical localization [11]. In addition, casein kinases have been shown as key regulators of the polarized expression of several proteins [28, 29, 40, 44]. For instance, CK1ε activity is required for proper localization of occludin in basolateral domains at tight junctions [29]. Moreover, CK2 activity is important in the basolateral localization of furin, mannose 6-phosphate receptor, and UT-A3 urea transporter [28, 40, 44]. Here, we show that the CK-mediated phosphorylation at TRPM4 S839 is required for proper polarized localization of TRPM4 channels on the basolateral plasma

membrane of epithelial cells. These data provide an additional function for this important protein kinase family.

TRPM4 has been shown to regulate several functions of the immune system, such as the secretion of interleukins by human T lymphocytes, the anaphylactic response of mast cells, and cell migration of dendritic cells [2, 23, 49]. The fundamental mechanism for this regulation is through changes in the concentration of intracellular calcium. It has been suggested that TRPM4 regulates calcium influx by depolarizing the membrane, favoring voltage-gated Ca^{2+} channel opening. In this study, we identified the S839 phosphorylation site as crucial to modulating the polarized localization of the TRPM4 channel. Therefore, TRPM4 phosphorylation may constitute a regulatory mechanism upstream of local changes in intracellular Ca^{2+} levels. Future work on the tissue specific phosphorylation and the signaling pathways involved in regulation of TRPM4 phosphorylation will be necessary to determine the physiological and pathophysiological regulation of TRPM4 localization and its role in determining intracellular Ca^{2+} levels.

Despite the lack of canonical motifs for basolateral localization in the primary sequence of TRPM4, it is clear that phosphorylation in the S839 residue regulates this process. Interestingly, the motif in which S839 residue is localized (^{837}GGpSLASGGP^{845}), resembles the AASLLAP motif of the NPP1 pyrophosphatase, which determines the basolateral localization but not endocytic regulation of this protein [4]. Our data demonstrate that S839 phosphorylation is similarly required for basolateral localization, but does not regulates its surface expression. We also demonstrated that CK1 regulates the TRPM4 basolateral localization in a S839-dependent manner. Therefore, we propose that this region of TRPM4 constitutes a non-canonical basolateral targeting motif whose function is regulated by CK-dependent phosphorylation. Future insights from the study of phosphorylation-dependent protein interactions will shed light on the mechanisms underlying dynamic regulation of TRPM4 channel localization and its physiological function.

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Author's contributions O.C., J.S.T., and A.S. designed research. O.C., M.C., K-S.P, E.L-S., and A.R. performed research. O.C., M.C., K-S.P, E.L-S, D.V., J.S.T, and A.S. analyzed data. O.C., J.S.T., and A.S. wrote the manuscript.

Conflict of interest The authors declare no conflict of interests.

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