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Regulation of calcium influx and signaling pathway in cancer cells via TRPV6–Numb1 interaction

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ABSTRACT

Ca^{2+} is a critical factor in the regulation of signal transduction and Ca^{2+} homeostasis is altered in different human diseases. The level of Ca^{2+} in cells is highly regulated through a diverse class of regulators. Among them is the transient receptor potential vanilloid 6 (TRPV6), which is a Ca^{2+} selective channel that absorbs Ca^{2+} in the small intestine. TRPV6 is overexpressed in some cancers and exhibits oncogenic potential, but its exact mechanism is still poorly understood. The Numb protein is a cell fate determinant that functions in endocytosis and as a tumor suppressor via the stabilization of p53. Numb protein consisted of four isoforms. Here, we showed a novel function of Numb1, which negatively regulates TRPV6 activity. The expression of Numb1 decreased cytosolic Ca^{2+} concentrations in TRPV6-transfected HEK293 cells. When all the isoforms of Numb were depleted using siRNA in a TRPV6 stable cell line, the levels of cytosolic Ca^{2+} increased. We observed an interaction between Numb1 and TRPV6 using co-immunoprecipitation. We confirmed this interaction using Fluorescence Resolution Energy Transfer (FRET). We identified the TRPV6 and Numb1 binding site using TRPV6 C-terminal truncation mutants and Numb1 deletion mutants. The binding site in TRPV6 was an aspartic acid at amino acid residue 716, and that binding site in Numb1 was arginine at amino acid residue 434. A Numb1 mutant, lacking TRPV6 binding activity, failed to inhibit TRPV6 activity. Every isoform of Numb knockdown, using an siRNA-based approach in MCF-7 breast cancer cells, not only showed enhanced TRPV6 expression but also both the cytosolic Ca^{2+} concentration and cell proliferation were increased. The down-regulated expression of TRPV6 using siRNA increased Numb protein expression; however, the cytosolic influx of Ca^{2+} and proliferation of the cell were decreased. To examine downstream signaling during Ca^{2+} influx, we performed Western blotting analysis on TRPV6 upregulated cancer cells (MCF-7, PC-3). Taken together, these results demonstrated that Numb1 interacts with TRPV6 through charged residues and inhibits its activity via the regulation of protein expression. Moreover, we provided evidence for a Ca^{2+}-regulated cancer cell signaling pathway and that the Ca^{2+} channel is a target of cancer cells.

1. Introduction

Ca^{2+} is a second messenger that strongly influences the activation or inhibition of several signaling pathways [1,3]. Ca^{2+} also plays a key role in the regulation of a number of proteins. The deregulation of Ca^{2+} homeostasis results in tumorigenesis, such as proliferation, angiogenesis, apoptosis, and gene transcription. Ca^{2+} homeostasis is thoroughly regulated via Ca^{2+} channels, pumps, and exchangers. Several cancers are closely connected with Ca^{2+} channels and pumps [2]. For example, Ca^{2+} channels TRPM8 and TRPV6 are upregulated in prostate cancer while the Ca^{2+} pump SERCA3 is downregulated in colon cancer [4–7].

Since the first Ca^{2+} channel TRP was cloned in *Drosophila*, 28 additional channels have been discovered in humans. Based on their structural homologies, these channels can be further divided into seven subfamilies (TRPV, TRPC, TRPM, TRPP, TRPA, TRPML, and TRPN). The TRPV (vanilloid) family has six members [8–10]. TRPV6 is a Ca^{2+} selective channel that contains a high sequence homology with TRPV5. TRPV6 is primarily expressed in the epithelial tissue

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of various organs such as the digestive tract, prostate, testis, placenta and skin. Among all TRP channels, TRPV5 and TRPV6 have the highest selectivity for calcium, making these channels important, especially in Ca^{2+}-related signaling pathways [11–13]. TRPM7 and TRPM8 are overexpressed in pancreatic cancer, and TRPC6 is overexpressed in liver, gastric, esophageal and glia cancers [14–19]. The expression of TRPV6 is substantially increased at both the mRNA and protein levels in tumor of several tissues in breast, prostate, colon, thyroid and ovary [20,21,5]. It has been reported that TRPV6 mediates the influx of Ca^{2+} entry into cytosol with the subsequent downstream activation of NFAT in LNCaP cells [22]. However, the mechanism underlying the TRPV6-mediated regulation of cancer progression and its downstream signaling remains unknown.

The Numb protein was first identified in *Drosophila* [23]. As a cell fate determinant that inhibits notch activity via endocytosis [24]. However, Numb was observed to show diverse functions besides Notch endocytosis. The Numb protein is involved in signal transduction pathways (Hedgehog, p53), endocytosis (cargo internalization and recycling), cell polarity determination, and ubiquitination [25]. In cancer, Numb functions as a tumor suppressor via the stabilization of p53 [26]. Numb forms a tricomplex with p53 and its negative regulator, MDM2. The loss of Numb expression has been observed in mammary and lung cancer [27,28]. Since Numb and Notch are crucial in tumorigenesis, it is expected that Notch and Numb modulate the aforementioned processes, in part, by modifying Ca^{2+}-sensitive signaling pathways. In previous study, it is reported that Numb regulates Notch-dependent expression of TRPC6 [40]. However it is hardly understood how Numb gives an influence on Ca^{2+} homeostatic mechanisms.

Using TRPV6 C-terminal as bait in a yeast two-hybrid screening, we have previously shown that Numb1 interacts with TRPV6 via yeast two hybrid screening [30]. In our current study, we showed that Numb1 interacts with TRPV6 via a charge–charge interaction that inhibits TRPV6 activity. We further demonstrated the association of Ca^{2+} with proliferation in cancer cells.

2. Materials and methods

2.1. Materials

Anti-c-myc (sc-40), anti-GFP (sc-8334) and anti-GSK3β (sc-7291) antibodies were obtained from Santa Cruz Biotechnology, Inc. The anti-TRPV6 (ACC-036) antibody was obtained from Alomone Labs. Protein G agarose beads (sc-2002) were obtained from Santa Cruz. Anti-AKT (#9272), anti-p-AKT (#4056), anti-p-JNK (#9251), anti-p38 (#9212), anti-p-p38 (#9216), anti-p-GSK3β (#9336), anti-Erk (#9102), anti-p-Erk (#9106) and anti-Numb (#2756) antibodies were obtained from cell signaling.

2.2. Cell culture and transfection

HEK293 cells were cultivated in DMEM (MCF-7 cells in RPMI) supplemented with 10% heat-inactivated FBS and penicillin (100 U/ml), streptomycin (100 μg/ml) at 37 °C in a 5% CO_2 humidified incubator. All plasmid DNAs used for transfection were prepared using a plasmid midi kit (QIAGEN) following the manufacturer's protocol. The transfection was performed with Fugene-6 according to the manufacturer's instructions.

2.3. Plasmid constructs

Numb1 cDNA was obtained from OriGene and subcloned into a pcDNA3-myc vector to generate a myc-fused Numb1 construct. The truncation mutants of Numb1, amino acids 9–651, 17–651, 25–651, 35–651, 1–639, 1–613, 1–600 and 1–570 were partially amplified using PCR and then subcloned into the EcoRI and XhoI restriction sites of pcDNA3-myc. To generate myc-fused Numb1 deletion constructs, isoform 2, 3, and 4, ΔPTB1, ΔPTB2, ΔPTB3, ΔPTB4, Δ176–220, Δ221–270, Δ271–320, Δ321–365, ΔDPF, ΔNPF, Δ541–570, Δ501–540, Δ461–500, and Δ414–460, we used site-directed mutagenesis with the QuikChange XL Site-Directed Mutagenesis Kit (Stratagene). The full length TRPV6 cDNA was subcloned into the pEYFP-C1 plasmid (Clontech) using the XhoI, and EcoRI restriction enzyme sites. The TRPV6 C-terminal truncation mutants, 1–721, 1–720, 1–719, 1–718, 1–717, 1–716, 1–715, 1–704, 1–680, 1–635, 1–617, 1–591 and 1–577, were PCR amplified and inserted into the same restriction sites of vector pEYFP-C1. All Numb and TRPV6 point mutation constructs were generated using site-directed mutagenesis.

2.4. Co-immunoprecipitation

HEK293 cells were plated in 60-mm dishes and cotransfected with myc and EYFP-tagged constructs. The Numb constructs were cotransfected with the TRPV6 constructs at a 1:1 ratio. After a 24 h incubation, the cells were harvested and lysed in 500 μl of cell lysis buffer (0.5% Triton X-100, 50 mM Tris–Cl pH 7.5, 150 mM NaCl, 1 mM EDTA). After clarification through centrifugation at 4 °C for 10 min at 15,000 × *g*, the whole cell lysates were incubated with 1 μg of anti-myc antibody and 25 μl of protein G-agarose beads at 4 °C overnight with gentle rotation. Subsequently, the beads were washed 3 times with wash buffer (0.1% Triton X-100, 50 mM Tris–Cl pH 7.5, 150 mM NaCl, 1 mM EDTA), and the precipitates were eluted with 30 μl 2× Laemmli buffer and subjected to Western blot analysis for anti-myc and anti-GFP immunoreactivity.

2.5. Immunoblotting

Aliquots (20–60 μg of protein) of the purified cell lysates, obtained with 0.5% Triton X-100 lysis buffer containing protease inhibitor cocktail (Calbiochem) and phosphatase inhibitor (Calbiochem), were clarified using centrifugation (15,000 × *g* for 10 min). The supernatants were heated for 1 min under reducing conditions and subsequently subjected to electrophoresis on an 8% SDS acrylamide gel. The proteins were transferred electrophoretically onto nitrocellulose membranes (BioRad), which were previously blocked in 5% (w/v) nonfat milk for 1 h, and further incubated with antibodies against TRPV6, Numb, and GAPDH overnight at 4 °C. After incubation with horseradish peroxidase-coupled secondary antibodies for 1 h at room temperature, the signal was detected using a commercial detection system (Thermo Scientific).

2.6. Measurement of intracellular Ca^{2+}

The ratiometric measurement of $[Ca^{2+}]_I$ was performed using Fura-2 AM (molecular probe). The cells were grown in 24-well dishes and loaded with 5 μM of Fura-2 AM for 30 min at 37 °C. The Fura-2 fluorescence was measured at a 510 nm emission with a 340/380 nm dual excitation using a DG-4 illuminator. The experiments were performed in an Normal Tyrode solution containing 3.6 mM KCl, 10 mM HEPES, 1 mM $MgCl_2$, 145 mM NaCl, 2 mM $CaCl_2$, 5 mM glucose, pH 7.4.

2.7. Fluorescence Resolution Energy Transfer

3 FRET images (cube settings for CFP, YFP, and Raw FRET) were obtained from a pE-1 Main Unit to 3 FRET cubes (excitation, dichroic mirror, filter) through a fixed collimator: CFP (ET435/20m, ET CFP/YFP/mCherry beamsplitter, ET470/24m, Chroma); YFP (ET500/20m, ET CFP/YFP/mCherry beamsplitter, ET535/30m,