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Identification of BSPRY as a Novel Auxiliary Protein Inhibiting TRPV5 Activity

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Transient receptor potential vallinoid 5 (TRPV5) and TRPV6 are the most Ca²⁺-selective members of the TRP superfamily and are essential for active Ca²⁺ reabsorption in epithelia. However, little is known about intracellular proteins that regulate the activity of these channels. This study identified BSPRY (B-box and SPRY-domain containing protein) as a novel factor involved in the control of TRPV5. The interaction between BSPRY and TRPV5 by GST pull-down and co-immunoprecipitation assays was demonstrated. BSPRY showed co-localization with TRPV5 in mouse kidney. Expression of BSPRY resulted in a significant reduction of the Ca²⁺ influx in Madin-Darby Canine Kidney cells that stably express TRPV5 without affecting channel cell-surface abundance. Finally, BSPRY expression in kidney was increased in 25-hydroxyvitamin D₃/1α-hydroxylase knockout mice, suggesting an inverse regulation by vitamin D₃. Together, these results demonstrate the physiologic role of the novel protein BSPRY in the regulation of epithelial Ca²⁺ transport via negative modulation of TRPV5 activity.


Materials and Methods

DNA Constructs and cRNA Synthesis

Full-length BSPRY was obtained from mouse expressed sequence tag (EST) L.M.A.G.E. 5116439, cloned into the pTLN oocyte expression vector (5) in frame with an amino-terminal VSV tag and subcloned into pGEX6p2 (Amersham Biosciences, Uppsala, Sweden) and pCB7 (6). Full-length and carboxyl-termini of TRPV5 and TRPV6 were obtained as described previously (7).

Yeast Two-Hybrid System

Y153 yeast strain transformation and subsequently screening of a mouse kidney cDNA library (Clontech, Palo Alto, CA) was performed as described previously (7).

Protein–Protein Interaction Assays

Glutathione S-transferase (GST) fusion proteins were expressed in Escherichia coli BL21 and purified according to the manufacturer’s protocol (Amersham Biosciences). BSPRY cRNA was synthesized in vitro using SP6 RNA polymerase as described previously (7) and injected into Xenopus laevis oocytes. After 2 d, oocytes were lysed in PBS that contained 0.4% (vol/vol) Triton X-100. [³⁵S]Methionine-labeled full-length TRPV5/TRPV6 protein was prepared using a reticulocyte lysate system. Xenopus oocyte lysates or in vitro–translated proteins were incubated with GST or GST-fusion proteins that were immobilized on glutathione-Sepharose 4B beads (Amersham Biosciences) in PBS that contained 0.4% (vol/vol) Triton X-100 for 2 h at room temperature, and bound proteins were visualized by autoradiography or immunoblotting. MDCK cells that were stably transfected with green fluorescent protein (GFP)-fused TRPV5 and VSV-BSPRY were lysed in sucrose buffer that fused contained 20 mM Tris (pH 7.4), 5 mM EDTA, 135 mM NaCl, 0.5% (vol/vol) NP-40, 0.2% (vol/vol) Triton X-100, and 10% (wt/vol) sucrose, incubated on ice for 60 min, and centrifuged for 30 min at 16,000 × g. Supernatants were incubated with guinea pig anti-TRPV5 or monoclonal anti-VSV antibodies (Sigma, St. Louis, MO).
immobilized on protein A-agarose beads (Kem-En-Tec A/S, Copenhagen, Denmark) for 16 h at 4°C. Immunoprecipitated proteins were analyzed by immunoblot analysis.

Reverse Transcription–PCR Analysis
Total RNA was isolated using TRIzol (Life Technologies/BRL, Life Technologies, Breda, The Netherlands). Total RNA (2 μg) was subjected to reverse transcription (RT) using Moloney murine leukemia virus reverse transcriptase, and a PCR for BSPRY was performed using the primers 5′-GATTCCGAGAATAAGC-3′ and 5′-GGGTGGATCAGACAGTG-3′. BSPRY mRNA expression was quantified by real-time PCR using SYBRGreen dye and shown as relative expression to mouse hypoxanthine-guanine phosphoribosyl transferase (detected with the forward primer 5′-TTATCAGAAGAGCTACTGAAATGATC-3′, reverse primer 5′-TTACCAGGTCAAATATTATCTCTCAAACAT-3′), which was used as an endogenous control to normalize variations in RNA extractions, the degree of RNA degradation, and variabilities in RT efficiencies. The primers that were used to amplify the forward primer 5′-ACCATTTCCTCTCAGCTGTG-3′ and 5′-GTATGCTCTTGGCTGTACCAC-3′.

Preparation of Antibodies and Immunohistochemistry
Rabbit anti-BSPRY antibodies were raised against the peptide H2N-LFPVFAVADQLISIV-COOH of mouse BSPRY and used for immunohistochemistry as described. For semiquantitative determination of protein levels, images were analyzed with Image J (http://rsb.info.nih.gov/ij/), resulting in quantification of the protein levels as the mean of integrated optical density as described (2). All negative controls, including preimmune serum and serum depleted of anti-BSPRY antibodies by incubation for 16 h with GST-BSPRY immobilized on glutathione-Sepharose 4B beads, were devoid of staining.

45Ca2+ Uptake Assay
Ca2+ uptake was determined using MDCK cells by incubation in uptake medium that contained 1.0 mM CaCl2 for 5 min at room temperature as described previously.

Cell-Surface Biotinylation
Proteins that were present at the cell surface of confluent stably transfected MDCK cells were biotinylated at 4°C using NHS-LC-LC-biotin (0.5 mg/ml; Pierce, Etten-Leur, The Netherlands), precipitated using neutrevin-coated beads (Pierce), and analyzed by immunoblot analyses.

Statistical Analyses
In all experiments, the data are expressed as mean ± SEM. Overall statistical significance was determined by ANOVA. P < 0.05 was considered significant.

Results and Discussion
Our study provides the first evidence of the physiologic role of BSPRY as a negative modulator of the epithelial Ca2+ channel TRPV5. This conclusion is based on four independent observations. First, the specific interaction between this channel and BSPRY was demonstrated by yeast two-hybrid, GST pull-down and co-immunoprecipitation assays. Second, BSPRY showed complete co-localization with TRPV5 in Ca2+-transporting tubular segments of the kidney. Third, stable expression of BSPRY significantly inhibited Ca2+ influx in confluent layers of MDCK cells expressing TRPV5. Fourth, BSPRY expression was inversely regulated by the calcitropic hormone vitamin D3.

Identification of BSPRY as a Novel TRPV5- and TRPV6-Associated Protein
To discover novel TRPV5- and TRPV6-associated proteins, we performed a yeast two-hybrid screen on a mouse kidney cDNA library. Using the mouse TRPV6 carboxyl-terminus as bait, five clones that were identified as BSPRY were isolated. BSPRY contains a B-box and SPRY domain, whose tentative functions are protein–protein interaction modules (8,9). The association of full-length BSPRY with GST-TRPV5 or TRPV6 carboxyl-termini, immobilized on Sepharose 4B beads, confirmed the yeast two-hybrid results and showed that TRPV5 (Figure 1A) also interacts with BSPRY (Figure 1A). Furthermore, the reverse approach, whereby full-length, GST-fused BSPRY was immobilized on Sepharose beads and incubated with in vitro–translated full-length TRPV5 or TRPV6, resulted in significant binding of both Ca2+-binding to BSPRY (Figure 1B). The binding of BSPRY was equal in the presence of 1 mM Ca2+ or in Ca2+-free (2 mM EDTA) conditions, demonstrating a Ca2+-independent association (data not shown). No interaction was observed with GST alone, indicating the specificity of the binding.

To substantiate further the BSPRY interaction, we generated MDCK cell lines that were stably transfected with GFP-fused TRPV5 and subsequently with VSV-BSPRY or the empty pcB7 vector. These cell lines allowed the investigation of TRPV5 in a polarized epithelium, whereas endogenous expression levels of these channels in native tissues, including kidney, were below the detection limit of immunoblot analysis. Furthermore, these polarized cells exhibit apical uptake of Ca2+ and provide a valuable model to study TRPV5-mediated Ca2+ influx (10). Using these stably transfected cells, TRPV5 could be co-immunoprecipitated with anti-VSV antibodies upon expression of VSV-BSPRY, as indicated by the specific bands at approximately 95 kD for the core-glycosylated GFP-TRPV5 and approximately 115 kD for complex-glycosylated GFP-TRPV5 (Figure 1C). TRPV5 was not detected in the immunoprecipitated sample in the absence of VSV-BSPRY, confirming the specificity of the procedure. Similarly, BSPRY was co-immunoprecipitated with TRPV5 in the reverse reaction using anti-TRPV5 antibodies that were immobilized on Sepharose beads, as represented by an immunopositive band of approximately 65 kD (Figure 1D). Here, β-actin was used as a control to demonstrate equal input of the samples. β-Actin was absent in the immunoprecipitated sample, indicating the specificity of the interaction.

Co-Localization of BSPRY and TRPV5
The expression of BSPRY in mouse tissues was analyzed by RT-PCR. BSPRY was detected in several tissues, including kidney, small intestine, prostate, lung, and uterus. BSPRY was less abundantly expressed in heart, whereas skeletal muscle and liver were negative (Figure 2A, top). All samples expressed β-actin, which was used as a positive control to confirm cDNA integrity (Figure 2A, bottom). Using anti-BSPRY antibodies, raised in rabbits using a conserved 15–amino acid peptide of the carboxyl-terminus of mouse BSPRY, we demonstrated that...
the localization of BSPRY in kidney is strikingly similar to the localization of TRPV5 (Figure 2B, bottom). BSPRY was present in the apical domain of all TRPV5-immunopositive tubules, previously identified as the second part of the distal convoluted tubule and connecting tubule (11,12). Proximal tubules and glomeruli were negative, and preimmune serum did not show any staining. Furthermore, immunopositive BSPRY staining was absent upon incubation of the serum with immobilized GST-BSPRY, whereas incubation with GST alone had no effect (Figure 2B, top). Similar co-localization with TRPV5 in distal convoluted tubule and connecting tubule was observed previously for calbindin-D28K and the Na+/H+ exchanger (12,13). These proteins play an essential role in renal transcellular Ca²⁺ transport as they respectively facilitate the diffusion of Ca²⁺ from the apical to the basolateral side and the extrusion into the blood. Similarly, the robust co-localization between BSPRY and TRPV5 strongly supports a physiologic and specific function of BSPRY in the regulation of epithelial Ca²⁺ transport by direct association with TRPV5 in the kidney.
BSPRY operates as a negative modulator of TRPV5 activity. (A) The expression level of GFP-tagged TRPV5 in stably transfected MDCK cells was determined by immunoblot using anti-GFP antibodies and shows equal expression levels in the presence or absence of BSPRY. (B) BSPRY expression was verified using monoclonal anti-VSV antibodies. (C) TRPV5 activity was determined by radioactive Ca\textsuperscript{2+} uptake in MDCK cells that were grown to confluence, demonstrating a significant inhibition of TRPV5-mediated Ca\textsuperscript{2+} influx upon BSPRY co-expression. (D) Intact GFP-TRPV5–expressing MDCK cells, stably transfected with pCB7-VSV-BSPRY or the empty pCB7 vector, were incubated with NHS-LC-LC-biotin to label cell-surface proteins. Biotinylated proteins were isolated using neutravidin-agarose beads, separated by SDS-PAGE, and TRPV5 was subsequently quantified by immunoblot analysis. TRPV5 plasma membrane abundance was equal in the presence or absence of BSPRY. TRPV5 and BSPRY were not detected in precipitates from cells that were not exposed to biotin, showing the specificity of the precipitation for biotinylated proteins. Similarly, BSPRY was detected in the plasma membrane by biotinylation (E) and immunocytochemistry (F, arrowheads) using anti-VSV antibodies on stably BSPRY-transfected MDCK cells. β-Actin was found in the lysate from the biotinylated MDCK monolayers but not in the fraction recovered from the neutravidin beads, demonstrating that intracellular proteins are not isolated using this procedure (data not shown). *P < 0.05 versus all. (G) The role of vitamin D\textsubscript{3} on BSPRY expression was determined by quantitative real-time PCR in kidneys of wild-type (WT; n = 11) or 25-hydroxyvitamin D\textsubscript{3}-1α-hydroxylase (1α-OHase) knockout mice. These knockout mice are unable to synthesize 1,25-dihydroxyvitamin D\textsubscript{3}, the biologic active form of vitamin D (4,19). Therefore, we assessed the expression of BSPRY in wild-type and 25-hydroxyvitamin D\textsubscript{3}-1α-hydroxylase (1α-OHase) knockout mice. These knockout mice are unable to synthesize 1,25-dihydroxyvitamin D\textsubscript{3}. (H) Effect of vitamin D\textsubscript{3} on BSPRY protein abundance in mouse kidney. Representative images showing kidney sections stained for BSPRY in WT or 1α-OHase KO mice. Protein abundance was determined by computerized analysis of immunohistochemical images and is presented as mean optical density (arbitrary units) for n = 24 to 30 pictures from five mice for each condition. *P < 0.05 versus WT.
thesize 1,25-dihydroxyvitamin D3 and are a valuable animal model to study vitamin D deficiency rickets type I (20). Quantitative real-time PCR showed significantly enhanced BSPRY mRNA expression in the 1α-OHase knockout mice compared with wild-type mice, suggesting that vitamin D negatively regulates the BSPRY expression (Figure 3G). Because the antibodies that we generated did not recognize BSPRY on immunoblot, the protein expression of BSPRY in these animals was semiquantified by immunohistochemistry. Depicted in Figure 3H are representative images of BSPRY expression in wild-type and 1α-OHase knockout mice. Computerized analysis of the immunohistochemical staining showed significantly enhanced BSPRY protein expression in the 1α-OHase knockout mice compared with wild-type mice, confirming the inverse regulation of BSPRY expression by circulating vitamin D on the protein level (Figure 3H). Together with the Ca2+ uptake results, this suggests that BSPRY operates as a negative modulator for TRPV5 and that this mechanism will be downregulated when vitamin D levels increase to stimulate active Ca2+ transport. These findings demonstrate a novel target for vitamin D in the regulation of active Ca2+ transport and provide new insight into the factors involved in the Ca2+-related (patho-)physiology.

Conclusion
We have identified BSPRY as a novel auxiliary protein of the epithelial Ca2+ channels. The association, co-localization, and functional analyses described in this study demonstrate the first physiologic role of the novel protein BSPRY in the direct regulation of TRPV5.

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