Methionine Sulfoxide Reductase B1 (MsrB1) Recovers TRPM6 Channel Activity during Oxidative Stress*

Received for publication, January 13, 2010, and in revised form, June 25, 2010 Published, JBC Papers in Press, June 28, 2010, DOI 10.1074/jbc.M110.103655

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Mg2+ is an essential ion for many cellular processes, including protein synthesis, nucleic acid stability, and numerous enzymatic reactions. Mg2+ homeostasis in mammals depends on the equilibrium between intestinal absorption, renal excretion, and exchange with bone. The transient receptor potential melastatin type 6 (TRPM6) is an epithelial Mg2+ channel, which is abundantly expressed in the luminal membrane of the renal and intestinal cells. It functions as the gatekeeper of transepithelial Mg2+ transport. Remarkably, TRPM6 combines a Mg2+-permeable channel with an α-kinase domain. Here, by the Ras recruitment system, we identified methionine sulfoxide reductase B1 (MsrB1) as an interacting protein of the TRPM6 α-kinase domain. Importantly, MsrB1 and TRPM6 are both present in the renal Mg2+-transporting distal convoluted tubules. MsrB1 has no effect on TRPM6 channel activity in the normoxic conditions. However, hydrogen peroxide (H2O2) decreased TRPM6 channel activity. Co-expression of MsrB1 with TRPM6 attenuated the inhibitory effect of H2O2 on TRPM6 channel activity (TRPM6, 67 ± 5% of control; TRPM6 + MsrB1, 81 ± 5% of control). Cell surface biotinylation assays showed that H2O2 treatment does not affect the expression of TRPM6 at the plasma membrane. Next, mutation of Met1755 to Ala in TRPM6 reduced the inhibitory effect of H2O2 on TRPM6 channel activity (TRPM6 M1755A: 84 ± 10% of control), thereby mimicking the action of MsrB1. Thus, these data suggest that MsrB1 recovers TRPM6 channel activity by reducing the oxidation of Met1755 and could, thereby, function as a modulator of TRPM6 during oxidative stress.

To maintain a physiological extracellular and intracellular Mg2+ concentration is of great importance to keep the accurate function of more than 300 enzymatic systems and the subsequent various biological and physiological processes (1–4). The kidney is the principal organ responsible for the regulation of the body Mg2+ balance. Around 80% of the total plasma Mg2+ is ultrafiltered through the glomeruli and subsequently reabsorbed passively in the proximal tubule and the thick ascending limb of Henle’s loop (5). The final urinary Mg2+ concentration is defined by active Mg2+ reabsorption in the distal convoluted tubule (DCT) (6).

The transient receptor potential melastatin type 6 (TRPM6) is a cation channel playing a crucial role in Mg2+ homeostasis. Mutations in TRPM6 cause hypomagnesemia with secondary hypocalcemia (7, 8). Interestingly, mice deficient of TRPM6 (TRPM6−/− mice) were essentially embryonically lethal, and the incidental TRPM6−/− mice that survived had neural tube defects (9). TRPM6 and its closest homologue TRPM7 uniquely combine an ion channel pore-forming region with a serine/threonine protein kinase domain. It is located at the carboxyl terminus and has similarities with members of the α-kinase family (10, 11). Previous studies demonstrated that receptor for activated C-kinase 1 (RACK1) and repressor of estrogen receptor activity (REA) interact with this domain and inhibit channel activity in an (auto)phosphorylation-dependent manner (12, 13). Moreover, modulation of TRPM6 channel activity by intracellular ATP requires the ATP-binding motif in the α-kinase domain (14). Although the phosphorylation activity of the TRPM6/7 α-kinase domains has been well determined, the role of these domains in regulating channel activity remains elusive (12, 15–18).

Over the last years, several studies have implicated TRPM channels in ischemia (19, 20). Sun et al. (21) showed that decreased TRPM7 channel expression significantly reduced neuronal cell death after global ischemia. Furthermore, TRPM4 channel activation in vascular smooth muscle has been shown to contribute to cell death of vascular cells during ischemic injury, and TRPM2 has been well studied in relation to oxidative stress (22–25). Accumulating evidence suggests that reactive oxygen species are not only harmful side products of cellular metabolism but also central players in cell signaling and regulation (26–29). Interestingly, renal DCT cells contain the largest number of mitochondria. However, the effect of oxidative stress on the epithelial Mg2+ channel TRPM6, expressed at the apical membrane of the DCT, has not been studied.

The aim of the present study was to investigate the role of the α-kinase domain in TRPM6 channel activity by the identification of associated proteins. To this end, the Ras recruitment system (RRS), a novel yeast two-hybrid screening system, which is designed to screen for partners of plasma membrane proteins, was applied (30). Here, we identified methionine sulfoxide reductase B1 (MsrB1) as a TRPM6-associated protein, bind-
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The standard pipette solution contained 150 mM NaCl, 10 mM EDTA, and 10 mM HEPES-NaOH, pH 7.2. The extracellular solution contained 150 mM NaCl, 10 mM HEPES-NaOH, pH 7.4, supplemented with 1 mM CaCl₂. To avoid breakdown, hydrogen peroxide (H₂O₂) was stored at 4 °C prior to use and added to the perfusate immediately (<1 min) prior to making recordings.

**EXPERIMENTAL PROCEDURES**

**Cell Culture and Transfection**—Human embryonic kidney (HEK) 293 cells were grown and transfected as described previously (31), and electrophysiological recordings were performed 48 h after transfection.

**DNA Constructs**—The α-kinase domain of mouse (1759–2028) TRPM6 was cloned into the pMet425-Myc-Ras (kind gift from Dr. A. Aronheim, Haifa, Israel) by PCR using mouse kidney cDNA as template. The α-kinase domain of human (1759–2022) TRPM6 was cloned into the pEBG vector using human TRPM6 in pCINeo/IRES-GFP (32) as template. Full-length mouse MsrB1 cDNA was cloned into pCR7 by PCR using mouse kidney cDNA and FLAG-tagged at the N-terminal tail. Wild-type human TRPM6 in the pCINeo/IRES-GFP vector was HA-tagged at the N-terminal tail as described previously (32). TRPM6 M1755A, TRPM6 M1775A, TRPM6 M1904A, and TRPM6 M1947A mutants were created using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s protocol. All constructs were verified by sequence analysis.

**RRS Screening**—RRS screening was performed as described previously (30). Briefly, cdc25-2 strains (kind gift from Dr. A. Aronheim) were co-transformed with the pMet425-Myc-Ras-TRPM6 α-kinase domain and mouse kidney cDNA library. Transformants were grown on selectable minimal glucose plates for 5 days at 25 °C followed by subsequent replica plating onto minimal galactose plates, incubating for 5–7 days at 37 °C. Library plasmids of positive colonies growing on galactose plates were isolated and further analyzed by DNA sequencing. The positive colonies were co-transformed with the pMet425-Myc-Ras-TRPM6 α-kinase domain into cdc25-2 cells to confirm specificity of interaction.

**RT-PCR**—Total RNA isolation from mouse tissue and reverse transcription were performed as described previously (33). MsrB1 and β-actin were amplified by PCR and subsequently analyzed by agarose gel electrophoresis.

**Electrophysiology**—Patch clamp experiments were performed in the tight seal whole-cell configuration at room temperature using an EPC-10 patch clamp amplifier computer controlled by the Pulse software (HEKA Elektronik, Lambrecht, Germany). Electrode resistances were 2–5 megohms, and capacitance and access resistance were monitored continuously. A ramp protocol, consisting of linear voltage ramp from −100 to +100 mV (within 450 ms), was applied every 2 s from a holding potential of 0 mV. Current densities were obtained by normalizing the current amplitude to the cell membrane capacitance. The time course of current development was determined by measuring the current at +80 and −80 mV. I/V relations were established from the ramp protocols. The analysis and display of patch clamp data were performed using Igor Pro software (WaveMetrics, Lake Oswego, OR). The standard pipette solution contained 150 mM NaCl, 10 mM EDTA, and 10 mM HEPES-NaOH, pH 7.2. The extracellular solution contained 150 mM NaCl, 10 mM HEPES-NaOH, pH 7.4, supplemented with 1 mM CaCl₂. To avoid breakdown, hydrogen peroxide (H₂O₂) was stored at 4 °C prior to use and added to the perfusate immediately (<1 min) prior to making recordings.

**Co-precipitation Assay**—HEK293 cells were transiently co-transfected with MsrB1 and pEBG-TRPM6-α-kinase or pEBG empty vector. 24 h after transfection, cells were lysed for 1 h on ice in lysis buffer (150 mM NaCl, 5 mM EDTA, 50 mM Tris-NaOH, pH 7.5, 0.33% (v/v) Triton X-100 including the protease inhibitors leupeptin (0.01 mg/ml), pepstatin (0.05 mg/ml), and phenylmethylsulfonyl fluoride (1 mM)). After centrifugation, supernatants of the lysates were incubated overnight with glutathione-Sepharose 4B beads at 4 °C. In the co-precipitation experiment with full-length TRPM6, HEK293 cells were co-transfected with pEBG-MsrB1 and TRPM6 pCIneo/IRES-GFP or pCIneo/IRES-GFP empty vector and subsequently lysed and incubated on glutathione-Sepharose 4B beads as described above. After extensive washing, the bound proteins were eluted with SDS-PAGE loading buffer. The co-precipitation was analyzed using the anti-MsrB1 antibody (kind gift from Dr. J. Moskovitz, Lawrence, KS), mouse anti-HA (Sigma) antibody, or anti-GST antibody (Sigma).

**Cell Surface Labeling with Biotin**—HEK293 cells, in poly-l-lysine (Sigma)-coated 10-cm dishes, were transiently transfected with 15 μg of HA-TRPM6. 72 h after transfection, cells were treated with 1 mM H₂O₂ for 10 min at 37 °C. Cell surface labeling with NHS-LC-LC-biotin (Pierce, Etten-Leur, The Netherlands) was performed as described previously (34). 1 h after homogenizing, biotinylated proteins were precipitated using NeutrAvidin-agarose beads (Pierce). TRPM6 expression was analyzed by immunoblot for the precipitates (plasma membrane fraction) and for the total cell lysates using the mouse anti-HA antibody.

**Immunoblotting**—Protein samples were denatured by incubation for 30 min at 37 °C in Laemmli buffer and then subjected to SDS-PAGE. Immunoblots were incubated with either mouse anti-HA or rabbit anti-MsrB1 antibody. Subsequently, blots were incubated with sheep horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG (Sigma) and then visualized using the enhanced chemiluminescence system.

**Immunohistochemistry**—Immunohistochemistry was performed as described previously (35). Briefly, mouse kidney sections were incubated for 16 h at 4 °C with rabbit anti-MsrB1 and guinea pig anti-TRPM6. To visualize TRPM6, tyramide signal amplification kit (PerkinElmer Life Sciences, Zaventem, Belgium) was used after incubation with biotin-coated goat anti-mouse secondary antibody. Images were taken with a Bio-Rad MRC 100 confocal laser scanning microscope.

**Statistical Analysis**—Values are expressed as mean ± S.E. Statistical significance between groups was determined by analysis of variance followed by Bonferroni’s multiple comparison test. Differences between the means of two groups were analyzed by an unpaired Student’s t test. p < 0.05 was considered statistically significant.
RESULTS

MsrB1 Interacts with TRPM6 α-Kinase Domain—To identify proteins interacting with the α-kinase domain of TRPM6, we applied the RRS. When compared with the conventional yeast two-hybrid screening system, RRS is more appropriate to detect interaction partners of plasma membrane proteins (30). In this approach, MsrB1, a methionine sulfoxide reductase (36), was identified as an interacting protein of the TRPM6 α-kinase domain. Subsequently, MsrB1 cDNA was co-transformed with the α-kinase domain of TRPM6 into cdc25-2 yeast strain to confirm the interaction. As shown in Fig. 1A, whereas the cdc25-2 strains co-transformed with MsrB1 and the TRPM6 α-kinase domain grow at 37 °C, yeast co-transformed with the control vector and TRPM6 α-kinase domain only survived at 24 °C. The association between TRPM6 and MsrB1 was further substantiated by co-precipitation studies of glutathione S-transferase (GST) and GST-TRPM6-kinase in MsrB1-expressing HEK293 cells. MsrB1 co-precipitated with the GST-α-kinase but not with GST alone (Fig. 1B, upper panel). MsrB1 was equally expressed in the tested conditions (Fig. 1B, lower panel). Furthermore, co-precipitation studies of full-length TRPM6 with MsrB1 in HEK293 cells showed that full-length TRPM6 associates with GST-MsrB1 but not with GST alone (Fig. 1C, upper panel). TRPM6 and MsrB1 were expressed in all conditions tested (Fig. 1C, middle and bottom panel).

MsrB1 Co-expresses with TRPM6 in Kidney—To address the tissue distribution of MsrB1, reverse transcription-polymerase chain reaction (RT-PCR) analysis was performed on a panel of mouse tissues. The expected DNA fragment of MsrB1 was detected in all tissues as indicated in Fig. 1D. The integrity of the cDNA was confirmed by the detection of β-actin. To further study the co-expression of MsrB1 and TRPM6 in kidney, immunohistochemistry was performed on serial mouse kidney sections. This analysis indicated 70% immunopositive staining for MsrB1 in the TRPM6-expressing DCT segment, which has been implicated in active Mg^{2+} reabsorption (32) (Fig. 1E).

H_{2}O_{2} Inhibits TRPM6 Channel Activity—Considering that MsrB1 is a stress protein that mainly exerts its function during oxidative stress (36), we hypothesized that MsrB1 regulates TRPM6 channel activity during oxidative stress. Therefore, we examined the effect of H_{2}O_{2} on TRPM6 channel activity. To
this end, HEK293 cells expressing TRPM6 were treated with 1 mM H₂O₂ during whole-cell patch clamp recordings. As shown in Fig. 2, A and B, H₂O₂ caused a significant inhibition of the TRPM6-mediated current (67 ± 5% of control, n = 13, p < 0.05) when compared with the non-treated cells (n = 11). Furthermore, we demonstrate that H₂O₂ inhibits TRPM6 channel activity in a dose-dependent effect with an IC₅₀ of 148 ± 26 μM (Fig. 2C).

\[ H₂O₂ \text{ Treatment Does Not Affect TRPM6 Cell Surface Expression} \text{—Next, the influence of } H₂O₂ \text{ on the amount of TRPM6 channels at the plasma membrane was investigated by cell surface biotinylation experiments. As shown in Fig. 2D (upper panel), treatment with } H₂O₂ \text{ did not affect the plasma membrane abundance of TRPM6. Of note, the protein levels of TRPM6 were equal in all tested conditions as verified by the total cell lysates (Fig. 2D, bottom panel).} \]

\[ MsrB1 \text{ Prevents the Inhibitory Effect of } H₂O₂ \text{—Next, the effect of MsrB1 on TRPM6 channel activity was investigated. Patch clamp analysis demonstrated that MsrB1 has no effect on TRPM6 channel activity (TRPM6 empty vector, 250 ± 42 pA/pF; TRPM6 + MsrB1, 224 ± 37 pA/pF, p > 0.2). To investigate the role of MsrB1 on TRPM6 channel activity during oxidative stress, TRPM6 and MsrB1 were co-expressed in HEK293 cells and treated with } H₂O₂ \text{ during whole-cell patch clamp recordings. Treatment with } H₂O₂ \text{ significantly decreased TRPM6 current (67 ± 5% of control, n = 13, p < 0.05), whereas co-expression of MsrB1 significantly attenuated this inhibition (81 ± 5% of control, n = 13, p < 0.05) (Fig. 3, A and B).} \]

\[ Involvement of Met₁⁷⁵⁵ in } H₂O₂\text{-inhibited TRPM6 Activity—Because MsrB1 binds to the TRPM6 α-kinase domain and recovers the inhibitory effect of } H₂O₂ \text{ on TRPM6 channel activity, we hypothesized that MsrB1 could reduce methionine oxidation of the α-kinase domain. Therefore, the methionine residues located on the periphery of the TRPM6 α-kinase domain three-dimensional structure,⁴ which might be more vulnerable to } H₂O₂ \text{, were mutated into alanines (namely M1755A, M1775A, M1904A, and M1947A as analyzed by Yasara software). Subsequent patch clamp analysis showed that only the TRPM6 M1755A mutant was not significantly inhibited by } H₂O₂ \text{ (83 ± 6% of control, n = 12, p > 0.05) (Fig. 4, A and B). Of note, the other mutants were significantly inhibited by } H₂O₂ \text{ (M1775A, 63 ± 10%; M1904A, 64 ± 9%; M1947A, 64 ± 7%; n = 10) (Fig. 4A).} \]

\[ \text{DISCUSSION} \]

In the present study, we identified MsrB1 as a new TRPM6-associated protein and showed that MsrB1 recovers TRPM6 activity during oxidative stress. MsrB1 binds to the TRPM6 α-kinase domain and recovers the inhibitory effect of H₂O₂ on TRPM6 channel activity, which might be due to the reduced methionine oxidation of the α-kinase domain. The identification of MsrB1 as a new TRPM6-associated protein opens new avenues for understanding the regulation of TRPM6 channel activity during oxidative stress.

\[ ⁴ \text{H. Venselaar, personal communication.} \]
channel activity via reducing the oxidation state of Met1755 during oxidative stress. First, MsrB1 directly binds to the TRPM6 α-kinase domain and co-precipitates full-length TRPM6. Second, MsrB1 is co-expressed with TRPM6 in the renal DCT. Third, H2O2 inhibits TRPM6 channel activity without affecting the plasma membrane expression. Fourth, MsrB1 prevents the inhibitory effect of H2O2 on TRPM6 channel activity. Finally, H2O2 has no significant effect on the TRPM6 M1755A mutant.

TRPM6 belongs to the TRPM subfamily of the TRP channels and is so far the only known channel directly mediating active transepithelial Mg2+ transport (4, 7, 8, 32). However, the molecular regulation of this channel remains elusive. Here, we used a novel yeast two-hybrid procedure, RRS, to screen proteins interacting with the TRPM6 α-kinase domain. Our data showed that MsrB1 binds to the α-kinase domain of TRPM6, resulting in the recruitment of Ras to the membrane and subsequent complementation of the temperature-sensitive cdc25-2 mutation, so we identified MsrB1 as a new interacting protein of the TRPM6 α-kinase domain. This interaction has been confirmed by a subsequent GST co-precipitation assay in HEK293 cells with full-length TRPM6. Importantly, MsrB1 and TRPM6 are co-expressed in the renal DCT, which further substantiates the physiological relevance of the interaction between both proteins.

MsrB1 is an oxidoreductase that catalyzes the thiol-dependent reduction of methionine sulfoxide (36). MsrB1 belongs to the Msr family composed of MsrA and MsrB. Mammals contain one MsrA and three MsrBs that are highly abundant in kidney, liver, heart, and nervous tissue (37–41). These enzymes protect cells from oxidative stress via the repair of oxidative damage to proteins and thereby restore biological activity. They can be involved in reactive oxygen species-mediated signal transduction through modulation of the function of target proteins (36, 42–44). Accumulating data showed that reversible methionine oxidation and reduction play a dynamic role in a variety of cellular signaling pathways (45, 46). For example, the methionine residues of Helix-3, Ca2+/calmodulin-dependent protein kinase II (CamKII), shaker voltage-dependent K+ channel, and Slo1 K+ channels can be oxidized and hereby regulate their function (46–49). It has been shown that oxidation of a methionine residue in the shaker voltage-dependent K+ channel disrupts its inactivation. This effect can be reversed by co-expression with MsrA1 (47).

In the present study, we showed that MsrB1 interacts with the TRPM6 α-kinase domain but does not affect the channel activity in normoxic conditions. However, H2O2 is a product during oxidative stress and has been studied in relation to potassium channel function and the TRPM2 channel (reviewed in Refs. 50 and 51). Here, we demonstrated that H2O2 significantly decreases the TRPM6-mediated current in HEK293 cells in a dose-dependent manner. As H2O2 did not change the surface expression of TRPM6, it possibly regulates TRPM6 channel activity directly through modulation of the channel conductance. Importantly, the decreased TRPM6 channel activity can be partly recovered by co-expression with MsrB1. This par-
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Many reports have demonstrated that oxidants such as hydrogen peroxide (H2O2) can modulate TRP channels. Recent studies have shown that oxidants inhibit the activity of TRP channels, which is critical for the regulation of channel activity and cell function. Among these oxidants, H2O2 is a major source of reactive oxygen species (ROS) generated from mitochondrial and macrophage sources.

In this study, we investigated the recovery of TRPM6 channel activity following H2O2 treatment. We found that Msrb1, a protein that interacts with TRPM6, is crucial for recovering TRPM6 channel activity. Msrb1 is a member of the metallothionein family and is expressed in the renal proximal tubule, where it is inhibited by H2O2. Therefore, it is proposed that Msrb1 modulates TRPM6 channel activity and plays a critical role in the regulation of Mg2+ reabsorption.

Acknowledgments—We greatly thank Dr. A. Aronheim for the RRS system and Dr. J. Moskovitz for the rabbit anti-Msrb1 antibody. We thank Dr. S. Verkaart for valuable discussion.

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