Regulation of TRP channel TRPM2 by the tyrosine phosphatase PTPL1

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Submitted 9 November 2006; accepted in final form 19 January 2007

Zhang W, Tong Q, Conrad K, Wozney J, Cheung JY, Miller BA. Regulation of TRP channel TRPM2 by the tyrosine phosphatase PTPL1. Am J Physiol Cell Physiol 292: C1746–C1758, 2007. First published January 24, 2007; doi:10.1152/ajpcell.00569.2006.—TRPM2, a member of the transient receptor potential (TRP) superfamily, is a Ca2+-permeable channel, which mediates susceptibility to cell death following activation by oxidative stress, TNFα, or β-amyloid peptide. We determined that TRPM2 is rapidly tyrosine phosphorylated after stimulation with H2O2 or TNFα. Inhibition of tyrosine phosphorylation with the tyrosine kinase inhibitors genistein or PP2 significantly reduced the increase in [Ca2+]i, observed after H2O2 or TNFα treatment in TRPM2-expressing cells, suggesting that phosphorylation is important in TRPM2 activation. Utilizing a TransSignal PDZ domain array blot to identify proteins which interact with TRPM2, we identified PTPL1 as a potential binding protein. PTPL1 is a widely expressed tyrosine phosphatase, which has a role in cell survival and tumorigenesis. Immunoprecipitation and glutathione-S-transferase pull-down assays confirmed that TRPM2 and PTPL1 interact. To examine the ability of PTPL1 to modulate phosphorylation or activation of TRPM2, PTPL1 was coexpressed with TRPM2 in human embryonic kidney-293T cells. This resulted in significantly reduced TRPM2 tyrosine phosphorylation, and inhibited the rise in [Ca2+]i, and the loss of cell viability, which follow H2O2 or TNFα treatment. Consistent with these findings, reduction in endogenous PTPL1 expression with small interfering RNA resulted in increased TRPM2 tyrosine phosphorylation, a significantly greater rise in [Ca2+]i following H2O2 treatment, and enhanced susceptibility to H2O2-induced cell death. Endogenous TRPM2 and PTPL1 was associated in U937-ecoR cells, confirming the physiological relevance of this interaction. These data demonstrate that tyrosine phosphorylation of TRPM2 is important in its activation and function and that inhibition of TRPM2 tyrosine phosphorylation reduces Ca2+ influx and protects cell viability. They also suggest that modulation of TRPM2 tyrosine phosphorylation is a mechanism through which PTPL1 may mediate resistance to cell death.

transient receptor potential channels; oxidative stress

THE TRANSIENT RECEPTOR POTENTIAL (TRP) superfamily is a diverse group of cation channels expressed in mammalian cells related to the archetypal Drosophila TRP (11, 19, 34). TRP superfamily members have been divided into six subfamilies (TRPC, TRPV, TRPM, TRPA, TRPP, and TRPML) based on sequence homology and are involved in many physiological processes, including vasoactivation, sensation, fertility, and cell proliferation (11, 34). Mammalian isoforms share several characteristics, including six transmembrane segments and are generally voltage independent. The TRPM subfamily was named after the first described member, melastatin (TRPM1), a putative tumor suppressor protein (12). Expression of TRPM1 in melanocytes correlated inversely with melanoma aggressiveness and the potential for metastasis. Other members of the TRPM family also have roles in cell proliferation or survival, including TRPM2 (14, 17, 55), TRPM5 (41), TRPM7 (1), and TRPM8 (5, 45).

TRPM2, also called LTRPC-2, was the second member of the TRPM family to be described (17, 36, 38, 42, 54). It is expressed in many cell types, including brain and hematopoietic cells (42). Extracellular signals that activate TRPM2 include oxidative stress, TNFα, amyloid β-peptide, and connexin43 (14, 16, 17, 50, 55). Stimulation with these extracellular signals is thought to result in production of intracellular ADP ribose, which activates TRPM2 by binding to the TRPM2 COOH-terminal NUDT9-H domain (16, 20, 27, 39, 40, 50). TRPM2 plays an important role in susceptibility to cell death induced by oxidative stress or TNFα, dependent on an increase in the free intracellular calcium concentration ([Ca2+]i) (14, 17, 55).

The protein tyrosine phosphatase-L1 (PTPL1) is a widely expressed, nonreceptor protein tyrosine phosphatase, which has an important role in cell survival and tumorigenesis (2, 23, 53). PTPL1, also called PTP-BAS (31), hPTP1E (4), or Fas-associated phosphatase-1 (FAP-1) (43), is one of the largest protein tyrosine phosphatases with 2,485 amino acids (3). It contains a kinase noncatalytic C-lobe domain and a four-point-one/ezrin/radixin/moesin domain in the NH2 terminus, five PDZ domains between residues 1102 and 1990, and a protein tyrosine phosphatase domain in the COOH terminus (10, 13, 48). The four-point-one/ezrin/radixin/moesin domain may be necessary for targeting of PTPL1 to the apical surface of the plasma membrane (13), and the PDZ domains play a role in regulating the intracellular localization of PTPL1 and its interaction with other substrates and proteins (22, 25, 26). Targets dephosphorylated by PTPL1 include phosphoephrin B ligands and proteins phosphorylated by Src kinases (13, 23, 49). The insulin receptor substrate protein and the phosphatidylinositol 3-kinase pathway are other targets of PTPL1 (6, 26, 48). The functional consequences of PTPL1-mediated dephosphorylation are, in general, not yet clear (13).

PTPL1 is highly expressed in several human tumors, and the level of PTPL1 expression correlates positively with resistance of tumors to FasL-mediated apoptosis (2, 23, 30, 32, 35, 46, 53). One mechanism through which PTPL1 may modulate tumorigenesis is through regulation of Fas cell surface expression. PTPL1 has been reported to directly interact with Fas, retaining Fas in cytoplasmic pools and inhibiting Fas cell surface expression (23). In Ewing’s sarcoma family tumors, the costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
which are characterized by the expression of the aberrant oncogenic transcription factor EWS-FLI1, high expression levels of PTPL1 result from transcriptional upregulation of PTPL1 by EWS-FLI1, with higher expression in metastatic compared with primary tumors (2). In contrast to the increased proliferation observed in cells overexpressing PTPL1, reduction in PTPL1 levels has been associated with enhanced susceptibility to apoptosis. Reduced PTPL1 expression in Ewing’s sarcoma family tumor cells resulted in increased sensitivity to etoposide-induced apoptosis in vitro (2). In addition, hematopoietic cells from patients with myelodysplastic syndromes have reduced or absent levels of PTPL1, which may contribute to the enhanced apoptotic death in cells from these patients’ bone marrows (35). These data suggest that the phosphatase PTPL1 has an important role in cell death and survival, and contributes to tumorigenesis.

Here, we demonstrate that the calcium-permeable channel TRPM2 is tyrosine phosphorylated within 1 to 10 min after treatment with H2O2, a model of oxidative stress, or TNFα, and that tyrosine phosphorylation modulates the rise in [Ca2+]i in TRPM2 expressing cells. PTPL1 associated with TRPM2, both endogenously and in transfected cells. Coexpression of PTPL1 with TRPM2 reduced tyrosine phosphorylation of TRPM2, blocked the rise in [Ca2+]i, and protected cells from death following H2O2 treatment. In contrast, reduction of endogenous PTPL1 expression resulted in increased TRPM2 tyrosine phosphorylation, enhanced [Ca2+]i, and increased susceptibility of cells to death. These data indicate that TRPM2 is a target for dephosphorylation by PTPL1 and suggest that modulation of TRPM2 phosphorylation and channel activation is a mechanism through which PTPL1 may mediate resistance to apoptosis.

EXPERIMENTAL PROCEDURES

Cell lines, cDNAs, and transfection methods. HEK-293T cells were cultured in Dulbecco’s modified Eagle’s media with 10% FBS. U937-ecoR cells, a cell line stably expressing ecotropic receptor for retrovirus and generated in the laboratory of Dr. Linda Penn (Ontario Cancer Institute, Toronto, Canada), were cultured in α-MEM with 10% FBS. TRPM2 was subcloned into pDNA3.1-V5/His TOPO vector and one of five different PTPL1-PDZ domains in pEBG-2T vector, which encodes an NH2-terminal tag. At 48 h posttransfection, cells were harvested and lysed in buffer (50 mM Tris, pH 7.5, 1 mM EDTA, 150 mM NaCl, pH 7.4, 0.5 M NaCl, and 0.1% Triton X-100). Western blot analysis was performed as described above. Band intensity was quantified with the use of a calibrated densitometer (model GS800, Bio-Rad) using Quantity One software.

Purification of GST-fusion proteins. HEK-293T cells were co-transfected with TRPM2 in pcDNA3.1-V5/His TOPO vector and one of five different PTPL1-PDZ domains in pEBG-2T vector, which encodes an NH2-terminal tag. At 48 h posttransfection, cells were harvested and lysed in buffer (50 mM Tris, pH 7.5, 1 mM EGTA, 150 mM NaCl, 0.5 M NaF, and phosphatase inhibitor cocktail (Complete Protease Inhibitor). Glutathione sepharose high-performance beads (Amersham Biosciences) were washed, lysate added to the beads, and the suspension was incubated for 30 min at 4°C. The beads were washed twice with lysis buffer supplemented with 0.5 M NaCl, and twice with buffer B (50 mM Tris, pH 7.5, 0.1 mM EDTA, and 0.1% β-ME) (26). Bound GST proteins were eluted with 50 mM Tris/10 mM reduced glutathione, pH 7.4, followed by Western blot analysis with anti-V5-HRP and anti-GST antibodies.

Downregulation of PTPL1 by siRNA. HEK-293T cells were co-transfected with TRPM2 in pcDNA3.1-V5/His TOPO vector and FAP (PTPL1) siRNA (sc-43560, Santa Cruz) or control siRNA (sc-37007, Santa Cruz). Transfection was performed following the manufacturer’s protocol using transfection reagents (sc29528) purchased from Santa Cruz. Transfection was verified by detection of specific PTPL1 transcripts in transfected cells.

Immuno blotting and immunoprecipitation. For Western blotting, whole cell lysates or immunoprecipitates were separated on 8% polyacrylamide gels, followed by transfer to Hybond-C Extra membranes (Amersham Biosciences, Piscataway, NJ). Western blot analysis was performed as previously described. Blots were incubated with anti-V5-HRP (1:10,000, Invitrogen), anti-phosphotyrosine (clone 4G10, 1:1,000, Upstate Cell Signaling, Lake Placid, NY), anti-PTPL1 (anti-FAP clone H300, 1:250 to 1:1,500, Santa Cruz Biotechnology, Santa Cruz, CA), anti-GST (1:10,000; Sigma, St. Louis, MO), anti-actin (1:10,000; Sigma), anti-tubulin (1:10,000; Sigma), and anti-TRPM2-C (1:1,000, Bethyl Laboratories, Montgomery, TX) antibodies. Blots were washed and incubated with the appropriate horseradish peroxidase (HRP)-conjugated antibodies (1:2,000). Enhanced chemiluminescence (ECL) was used for detection of signal. To examine TRPM2 tyrosine phosphorylation or interaction of TRPM2 and PTPL1, immunoprecipitation was performed. Cells were washed in ice-cold Hanks’ balanced salt solution and lysed in buffer (50 mM Tris, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100) supplemented with Complete Protease Inhibitor Cocktail (Roche). For phosphorylation studies, 10 mM NaF and phosphatase inhibitor cocktail 2 (Sigma) were added into lysis buffer. Proteins were immunoprecipitated by mixing lysates with anti-V5 antibody (2 μg/ml lysate, Invitrogen) and protein G-sepharose 4B Fast Flow beads (Sigma) for 4 h at 4°C, or anti-TRPM2-C antibody (6 μg/ml lysate) overnight at 4°C, followed by addition of protein A/G PLUS-agarose beads (Santa Cruz). Immunoprecipitates were washed three times, and sample buffer was added to the pellets. For immunoprecipitation of FLAG-PTPL1, cell lysates were preabsorbed with protein A-sepharose GL-4B (Amersham Biosciences) and immunoprecipitated with 50 μl anti-FLAG M2 affinity gel (Sigma) for 2 h at 4°C. Samples were washed three times, and peptide elution was performed by the addition of 40 μl FLAG peptide (at 0.5 mg/ml; Sigma). Immunoprecipitates were heated at 60°C for 30 min to prevent aggregation before gel entry, which has been reported for membrane proteins (15). Western blot analysis was performed as described above. Band intensity was quantified with the use of a calibrated densitometer (model GS800, Bio-Rad) using Quantity One software.
the presence of Pluronic F-127. The extracellular buffer routinely contained 0.68 mM CaCl₂. In some experiments, cells were pretreated for 30 min and during the experiment with the tyrosine kinase inhibitor genistein (Calbiochem, La Jolla, CA), its inactive analog daidzein (Calbiochem), the Src kinase inhibitor PP2 or its negative control PP3 (Calbiochem). HEK-293T cells were then treated with 0, 1 mM H₂O₂, or 100 ng/ml TNFα. [Ca²⁺]ᵢ was measured in individual cells at baseline and at 1- to 2-min intervals for 20 min by determination of the fluorescence intensity ratio R (F₄₄₀/F₉₀₀). Where indicated, [Ca²⁺]ᵢ was measured more frequently during the first 2 min. The constants S₀₁, S₀₂ and the Kᵣ of Fura red were calibrated and Rₘᵢₙ and Rₚₐ₉ max measured for Fura Red as described previously (9). [Ca²⁺]ᵢ was calculated using the formula [Ca²⁺]ᵢ = Kᵣ[₀1 + (Rᵢ – Rₘᵢₙ)/(Rₚₐ₉ₙ – Rᵢ)] (S₀₁/S₀₂). Statistical significance of results was analyzed with one-way ANOVA.

Assays of cell viability. Cell viability was assessed by trypan blue exclusion. Apoptosis was assessed using the Vibripoint Apoptosis Assay Kit no. 2 (Molecular Probes) following the manufacturer’s protocol. Apoptotic cells labeled with annexin V conjugated to Alexa Fluor 488 and dead cells labeled with propidium iodide (PI) were detected with fluorescence microscopy using a Nikon Eclipse TE2000 inverted microscope and CoolSnap HQ Monochrome Camera.

RESULTS

TRPM2 is tyrosine phosphorylated following treatment with H₂O₂ or TNFα. The activity of several TRP channels is regulated by tyrosine phosphorylation (21, 24, 47), but the role of phosphorylation in TRPM2 activation is not known. We first examined whether TRPM2 is tyrosine phosphorylated following treatment with H₂O₂ or TNFα. HEK-293T cells heterologously expressing V5-tagged TRPM2 were treated with 1 mM H₂O₂ or 100 ng/ml TNFα for 0 to 60 min. TRPM2 was immunoprecipitated with anti-V5 antibody, and Western blots were probed with anti-phosphotyrosine and anti-V5-HRP antibodies. Representative results of five experiments with H₂O₂ and four experiments with TNFα are shown in Fig. 1. Baseline TRPM2 phosphorylation was low or absent. Significant tyrosine phosphorylation of TRPM2 was observed at 1 to 10 min after treatment with H₂O₂ or TNFα, which returned to baseline by 30 min. A similar increase in TRPM2 tyrosine phosphorylation was observed following treatment with 250 μM H₂O₂ (not shown).

Extracellular signals, including oxidative stress and TNFα, stimulate Ca²⁺ influx through TRPM2, resulting in a large and sustained increase in [Ca²⁺]ᵢ (17, 44, 50, 54). Representative time courses of the change in [Ca²⁺]ᵢ following treatment of HEK-293T cells transfected with empty vector (BFP-V) or BFP-TRPM2 with vehicle or H₂O₂ are shown in Fig. 2, A–D. The peak increase in [Ca²⁺]ᵢ occurred after 10–20 min of treatment with H₂O₂ or TNFα, consistent with previously published results (14, 17, 40, 54, 55). To determine whether tyrosine phosphorylation is important in TRPM2 channel activation, we examined the effect of inhibition of endogenous tyrosine kinases on the rise in [Ca²⁺]ᵢ observed in TRPM2-expressing cells after treatment. We utilized a system we established to quantitate [Ca²⁺]ᵢ in single cells (8). HEK-293T cells were transfected with empty vector or BFP-TRPM2 in pQBI50. Successfully transfected individual cells were identified by detection of BFP. Some groups of cells were pretreated with the broad tyrosine kinase inhibitor genistein or its inactive analog daidzein. [Ca²⁺]ᵢ was measured in Fura red-loaded cells before and at 1- to 2-min intervals for 20 min after treatment with vehicle (PBS), 1 mM H₂O₂, or 100 ng/ml TNFα. The mean percent increase in [Ca²⁺]ᵢ, for each experimental group following treatment was calculated after comparing the peak increase in [Ca²⁺]ᵢ, for an individual cell to its baseline. H₂O₂ or TNFα stimulated an increase in [Ca²⁺]ᵢ, which was significantly greater in HEK-293T cells expressing BFP-TRPM2 than in cells transfected with empty pQBI50 (BFP-V) vector (P < 0.001, P < 0.05, respectively; Fig. 3). This is consistent with previous reports (17, 55). In five experiments, genistein pretreatment significantly inhibited the rise in [Ca²⁺]ᵢ observed in TRPM2-expressing cells in response to H₂O₂ or TNFα (P < 0.001, P < 0.01, respectively), whereas daidzein did not (Fig. 3). To further examine whether a Src kinase is involved, cells were pretreated with 4 μM of the Src-specific inhibitor PP2 or its negative control PP3. PP2 but not PP3 significantly inhibited the rise in [Ca²⁺]ᵢ in TRPM2-expressing cells following treatment with H₂O₂ or TNFα (P < 0.001, P < 0.001, respectively; Fig. 3). These results demonstrate that tyrosine phosphorylation has a major role in TRPM2 activation. Endogenous TRPM2 protein was only weakly detected by Western blot analysis in HEK-293T cells after long exposure times. In addition, the endogenous calcium response to H₂O₂ or TNFα in cells transfected with empty vector was not significantly inhibited by genistein or PP2 (data not shown), suggesting that the majority of the increase in [Ca²⁺]ᵢ in nontransfected HEK-293T cells may be secondary to activation of other channels.

TRPM2 interacts with the tyrosine phosphatase PTPL1. To identify candidate proteins that interact with TRPM2 and modulate its function, we initially utilized a TransSignal PDZ Domain Array I blot from Panomics (Redwood City, CA). PDZ domains are regions of sequence with homology found in diverse signaling proteins. PDZ recognition sequences are often present in transmembrane receptors and channels, and modulate interactions with proteins which express PDZ domains (18, 37). The TransSignal PDZ Domain Array I blot has 32 protein sequences with PDZ domains spotted on an array
membrane, including the first and fifth PDZ domains of PTPL1. The blot was incubated with lysates from HEK-293T cells expressing V5-TRPM2, followed by exposure to anti-V5-HRP antibody and ECL. V5-TRPM2 interacted with the first and fifth PDZ domains of PTPL1 (data not shown). These data suggested that the tyrosine phosphatase PTPL1 may interact with TRPM2 and therefore may play a role in TRPM2 activation by modulation of tyrosine phosphorylation.

![Fig. 2](image-url)

Fig. 2. Representative time courses of intracellular Ca\(^{2+}\) concentration ([Ca\(^{2+}\)\(_i\)]) after H\(_2\)O\(_2\) treatment. Fura red-loaded HEK-293T cells were transfected with (A and B) empty vector (BFP-V), (C and D) BFP-TRPM2 in pQBI50, or (E and F) BFP-TRPM2 and PTPL1 in pTracer-CMV. Cells were treated at time 0 with vehicle (PBS) or 1 mM H\(_2\)O\(_2\). [Ca\(^{2+}\)\(_i\)] was measured in representative cells at baseline, at 5-s intervals for the first 30 s, at 15-s intervals for the next 90 s, and then at 2-min intervals to 20 min.

![Fig. 3](image-url)

Fig. 3. Tyrosine kinase inhibitors block TRPM2 activation. HEK-293T cells were transfected with empty pQBI50 vector (BFP-V) or TRPM2 in pQBI50. At 48 h, single transfected cells were identified by BFP fluorescence with digital video imaging (DVI). [Ca\(^{2+}\)\(_i\)] was measured in Fura red-loaded BFP-expressing cells at baseline and at 1- to 2-min intervals during 20 min of treatment with PBS, 1 mM H\(_2\)O\(_2\), or 100 ng/ml TNF\(_\alpha\). Where indicated, cells were treated for 30 min before and during treatment with genistein (G; 50 \(\mu\)M), its inactive analog daidzen (D; 50 \(\mu\)M), the Src inhibitor PP2 (4 \(\mu\)M), or its negative control PP3 (4 \(\mu\)M). Results from five experiments were combined and 15 to 39 individual cells were studied in each group. The means ± SE % increase in [Ca\(^{2+}\)\(_i\)] above baseline represents the peak [Ca\(^{2+}\)\(_i\)] measurement obtained during monitoring over 20 min divided by baseline × 100% − 100% (baseline). ** \(p < 0.01\), significant differences between specified groups.
To confirm that TRPM2 associates with PTPL1, immunoprecipitation experiments were performed on lysates from HEK-293T cells expressing V5-tagged TRPM2, FLAG-tagged PTPL1, or both. With the use of anti-FLAG M2 affinity gel or anti-V5 antibody, TRPM2 was immunoprecipitated reciprocally with PTPL1 (Fig. 4A), suggesting that TRPM2 and PTPL1 interact. This experiment was performed eight times with similar results. Of note, with very long exposure times, endogenous PTPL1 could be detected in lysates (Fig. 4A, b). To determine whether PTPL1 and TRPM2 interact endogenously, lysates were prepared from the human monocytic leukemia cell line U937-ecoR, which expresses higher levels of endogenous TRPM2 protein than HEK-293T cells (55). TRPM2 was immunoprecipitated with anti-TRPM2-C antibody, and Western blotting of immunoprecipitates and supernatants was performed with anti-PTPL1 and anti-TRPM2-C antibodies. Although long exposure times were required to see endogenous PTPL1 protein, PTPL1 did immunoprecipitate with TRPM2 (Fig. 4B). Neither protein was observed in supernatants, likely because the low concentrations of these endogenous proteins in the supernatant made detection difficult (not shown). Neither PTPL1 nor TRPM2 precipitated with normal rabbit serum, showing specificity of results. Coimmunoprecipitation was observed in four experiments, demonstrating that these two endogenous proteins associate.

To further characterize the interaction of PTPL1 with TRPM2, we examined the ability of each of the five isolated PDZ domains of PTPL1 to interact with TRPM2 using a GST pull-down assay. The five PTPL1 PDZ domains located between residues 1099–1199, 1372–1467, 1506–1615, 1796–1887, and 1888–1990 were individually subcloned into pEBG2T, expressing PDZ domains with a GST tag (26). HEK-293T cells were transfected with V5-TRPM2 and each of these GST-fusion proteins, and GST pull-down performed. The first and fifth GST-tagged PDZ domains of PTPL1 bound to V5-TRPM2, whereas in parallel experiments the second, third, and fourth PDZ domains of PTPL1 failed to bind (Fig. 5). These results confirm the findings of immunoprecipitation experiments demonstrating interaction of TRPM2 and PTPL1, and the TransSignal PDZ Domain Array Blot I, indicating interaction of TRPM2 with PDZ domains 1 and 5. This experiment was repeated three times with identical results.

Increased expression of PTPL1 reduces TRPM2 tyrosine phosphorylation. As TRPM2 and PTPL1 associate, we examined whether PTPL1 is involved in regulation of TRPM2 tyrosine phosphorylation. HEK-293T cells transfected with V5-TRPM2 in the presence or absence of FLAG-PTPL1 were treated for 0 to 30 min with 1 mM H2O2 or 100 ng/ml TNFα. TRPM2 was immunoprecipitated from lysates with anti-V5 antibody, followed by Western blot analysis with anti-phosphotyrosine, anti-V5, or anti-PTPL1 antibodies. Tyrosine phosphorylation of TRPM2 observed after H2O2 or TNFα treatment was reduced in the presence of cotransfected PTPL1, suggesting that TRPM2 is a target for dephosphorylation by this phosphatase. A representative result of three experiments is shown in Fig. 6. Densitometry was used to quantitate tyrosine phosphorylated and total V5-TRPM2 bands. The amount of phosphorylated TRPM2 was normalized to the amount of total TRPM2 immunoprecipitated at each time point, and the mean ± SE %phosphorylation of TRPM2 in the...
that TRPM2 levels were not decreased in cells coexpressing TRPM2 and PTPL1 compared with other groups of cells expressing TRPM2, which could explain differences between groups in the [Ca\textsuperscript{2+}]\textsubscript{i} response after treatment. As shown in Fig. 7, the amount of TRPM2 expressed in PTPL1 cotransfected cells was similar to that expressed in cells transfected with TRPM2 alone or TRPM2 with empty pTracer-CMV vector. The higher molecular mass of TRPM2 in Fig. 7 (200 kDa) compared with that observed in other experiments (171 kDa) is secondary to linkage of TRPM2 to BFP. This experiment was repeated twice with similar results.

To assess the role of PTPL1 in mediating susceptibility to cell death through TRPM2 (17, 55), HEK-293T cells transfected with V5-TRPM2, FLAG-PTPL1, empty vectors, or combinations were treated with 1 mM H\textsubscript{2}O\textsubscript{2}. Viability was assessed by trypan blue exclusion and Annexin V staining. At 1, 6, and 24 h after treatment, cell viability assessed by trypan blue exclusion was significantly reduced in cells expressing TRPM2 and empty pFLAG-CMV2 vector (vector 1), compared with nontransfected cells (P < 0.001) or cells expressing PTPL1 and pcDNA3.1/V5-His TOPO vector (vector 2; P < 0.001) (see Fig. 8A). Coexpression of PTPL1 with TRPM2 preserved cell viability compared with cells expressing TRPM2 and empty vector 1 (P < 0.01) (Fig. 8A). Viability of cells expressing PTPL1 and empty vector 2 was equivalent to

The presence of PTPL1 compared with its absence was calculated from three experiments. The amount of TRPM2 that was tyrosine phosphorylated in cells coexpressing PTPL1 was 24 ± 4% (P < 0.0001) of that phosphorylated in cells transfected with V5-TRPM2 alone at 5 min after H\textsubscript{2}O\textsubscript{2} treatment and 31 ± 16% (P < 0.015) at 10 min after H\textsubscript{2}O\textsubscript{2} treatment. A similar significant reduction in TRPM2 phosphorylation was observed in PTPL1 coexpressing cells following TNF\textalpha treatment (P < 0.001).

Overexpression of PTPL1 in TRPM2-expressing cells reduces Ca\textsuperscript{2+} influx and susceptibility to cell death. The ability of PTPL1 to regulate TRPM2 activation was explored in HEK-293T cells transfected with BFP-TRPM2, PTPL1 in pTracer-CMV, empty pQBI50 (BFP-V), empty pTracer-CMV (GFP-V), or combinations. Successfully transfected individual cells were identified by detection of GFP and BFP. [Ca\textsuperscript{2+}]\textsubscript{i} was measured in Fura red-loaded cells after treatment with vehicle (PBS) or 1 mM H\textsubscript{2}O\textsubscript{2}. Cells transfected with BFP-TRPM2 alone or BFP-TRPM2 with empty GFP-V vector demonstrated a significantly greater increase in [Ca\textsuperscript{2+}]\textsubscript{i} after treatment with H\textsubscript{2}O\textsubscript{2} (268 ± 18%, 254 ± 12%, respectively; P < 0.001) than cells transfected with both BFP-TRPM2 and PTPL1 (131 ± 9%; Table 1). Representative time courses of the change in [Ca\textsuperscript{2+}]\textsubscript{i}, following treatment of HEK-293T cells transfected with BFP-TRPM2 and PTPL1 with vehicle or H\textsubscript{2}O\textsubscript{2} are shown in Fig. 2, E and F. The increase in [Ca\textsuperscript{2+}]\textsubscript{i} in cells transfected with BFP-TRPM2 and PTPL1 was not significantly different from the increase in cells transfected with empty BFP-V vector (129 ± 16%), empty BFP-V and GFP-V vectors (132 ± 18%), or empty BFP-V vector and PTPL1 (95 ± 10%). These studies demonstrate that PTPL1 inhibits the increase in [Ca\textsuperscript{2+}]\textsubscript{i}, following TRPM2 activation and suggest that regulation of TRPM2 tyrosine phosphorylation by PTPL1 may modulate Ca\textsuperscript{2+} influx. Western blot analysis was performed to establish
TRPM2 (was significantly less than that of cells transfected with 
P/H11006 empty pFLAG-CMV2 vector demonstrated significantly more 
treated with 1 mM H2O2 for 6 h. When HEK-293T cells were 
transfected with both TRPM2 and PTPL1, the number of 
protein; TRPM, member of the transient receptor potential family. Fura 
triazole (GFP-V), pTracer-CMV expressing PTPL1 or combinations. Cells were 
treated with PBS or 1 mM H2O2 for 20 min. [Ca2 
i was measured at baseline, 
and at 1- to 2-min intervals over 20 min. %Increase above baseline represents 
time 0 
4% in cells transfected with siRNA targeted to PTPL1 
was down regulated was observed in all four experiments. A 
significant difference between the two groups was determined 
by analysis of paired means over the time period of measurement (P = 0.001). PTPL1 expression was also quantitated by 
densitometry measurements and was reduced to a mean of 
55 ± 4% in cells transfected with siRNA targeted to PTPL1 
compared with control siRNA. These results indicate that 
induced in TRPM2-expressing cells through both apoptotic and necrotic processes.  
Downregulation of endogenous PTPL1 with siRNA in 
creases TRPM2 tyrosine phosphorylation, Ca2 
 influx, and 
susceptibility to cell death. To further examine the physiological significance of these findings, HEK-293T cells were de 
pleted of endogenous PTPL1 by cotransfection with TRPM2 and 
siRNAs targeted to PTPL1. Western blot analysis demonstrated 
reduction of endogenous PTPL1 protein expression by 
siRNA targeted to PTPL1 (Fig. 9A). No reduction in PTPL1 
was observed in cells transfected with control siRNA. Longer 
exposure times were required to see endogenous PTPL1 protein 
bands, compared with that used to visualize the PTPL1 band in transfected cells. For cells in which PTPL1 expression 
was downregulated by siRNA, both TRPM2 and actin expression 
were unaffected (Fig. 9A). This experiment was repeated 
four times with similar results. 
To examine the effect of depletion of endogenous PTPL1 on 
TRPM2 phosphorylation, HEK-293T cells were cotransfected with V5-TRPM2 and control siRNA or siRNA targeted to 
PTPL1. Cells were treated with 1 mM H2O2 for 20 min, and 
lysates from transfected cells in each group were subjected to Western 
blot analysis with anti-V5 antibody and Western blot analysis with anti-phosphotyrosine or anti-V5-HRP antibodies. A representative Western blot from one of four experiments is shown in Fig. 9B, demonstrating 
increased tyrosine phosphorylation of TRPM2 in cells transfected with siRNA targeted to PTPL1. Tyrosine phosphoryl 
ated TRPM2 bands were quantitated with densitometry and 
normalized to densitometry measurements of V5-TRPM2 bands. Baseline phosphorylation (100%) was that detected at 
time 0 in cells transfected with V5-TRPM2 and control siRNA. The mean ± SE %increase in phosphorylated/total TRPM2 
above baseline after treatment with H2O2 was calculated for four 
experiments, and results are shown in Fig. 9C. Increased 
tyrosine phosphorylation of TRPM2 in cells in which PTPL1 
was downregulated was observed in all four experiments. A 
significant difference between the two groups was determined 
by analysis of paired means over the time period of measurement (P = 0.001). PTPL1 expression was also quantitated by 
densitometry measurements and was reduced to a mean of 
55 ± 4% in cells transfected with siRNA targeted to PTPL1 
compared with control siRNA. These results indicate that 

Table 1. [Ca2+]i, response of HEK-293T cells expressing TRPM2 and PTPL1

<table>
<thead>
<tr>
<th>Vector</th>
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<th>Stimulation</th>
<th>Baseline</th>
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<tr>
<td>BFP-V</td>
<td>11</td>
<td>PBS</td>
<td>32±2</td>
<td>45±2</td>
<td>44±10*</td>
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Values are means ± SE, n, number of cells studied. HEK, human embryonic kidney; [Ca2 
i], intracellular Ca2 
concentration; GFP, green fluorescent protein; TRPM, member of the transient receptor potential family. Fura 
edred-loaded HEK-293T cells were transfected with empty pQB50 vector (BFP-V), pQB50 expressing BFP-TRPM2, empty pTracer-CMV vector (GFP-V), pTracer-CMV expressing PTPL1 or combinations. Cells were 
treated with PBS or 1 mM H2O2 for 20 min. [Ca2 
6 h after treatment by labeling cells with Alexa Fluor 488 
annexin V conjugates; annexin V binds to phosphatidylserine 
6 h after treatment with 1 mM H2O2 for 6 h is 
shown in Fig. 8C. Minimal apoptosis or necrosis was observed in 
nontransfected HEK-293T cells (4 ± 1% labeled with 
annexin V, 5 ± 1% labeled with PI, 1,197 total cells counted) 
or cells transfected with PTPL1 (4 ± 1% labeled with annexin 
V, 4 ± 1% labeled with PI, 1,232 cells counted). In contrast to 
these cells, HEK-293T cells transfected with TRPM2 and 
empty pFLAG-CMV2 vector demonstrated significantly more 
apoptosis and necrosis (14 ± 2% labeled with annexin V, 19 ± 3% labeled with PI, 1,130 cells counted; P ≤ 0.001) when 
treated with 1 mM H2O2 for 6 h. When HEK-293T cells were 
transfected with both TRPM2 and PTPL1, the number of 
apoptotic and necrotic cells (8 ± 2% labeled with annexin V, 
6 ± 1% labeled with PI, 1,288 cells counted) after treatment 
was significantly less than that of cells transfected with 
TRPM2 (P < 0.001) but not significantly different from 
nontransfected cells or cells expressing PTPL1 alone. These 
results confirm a role for PTPL1 in modulating cell death 

nontransfected cells. Four experiments showed similar results. Western blot analysis of lysates from transfected cells used in 
viability studies confirmed equivalent expression levels of 
heterologous TRPM2 or PTPL1 in the different experimental 
groups (Fig. 8B).

To assess the ability of PTPL1 to inhibit apoptosis or 
necrosis induced through TRPM2 in response to oxidative 
stress, HEK-293T cells transfected with TRPM2, PTPL1, or 
both were treated with 1 mM H2O2. Apoptosis was assessed at 
6 h after treatment by labeling cells with Alexa Fluor 488 
annexin V conjugates; annexin V binds to phosphatidylserine 
on the surface of early apoptotic cells. Necrosis was assessed 
by labeling with PI, which binds to nucleic acids in necrotic 
cells but does not penetrate the membrane of live or early 
apoptotic cells. Two experiments were performed and cell 
viability counted in triplicate. A representative field for each 
group of cells following treatment with 1 mM H2O2 for 6 h is 
shown in Fig. 8C. Minimal apoptosis or necrosis was observed in 
nontransfected HEK-293T cells (4 ± 1% labeled with 
annexin V, 5 ± 1% labeled with PI, 1,197 total cells counted) 
or cells transfected with PTPL1 (4 ± 1% labeled with annexin 
V, 4 ± 1% labeled with PI, 1,232 cells counted). In contrast to 
these cells, HEK-293T cells transfected with TRPM2 and 
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6 ± 1% labeled with PI, 1,288 cells counted) after treatment 
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TRPM2 (P < 0.001) but not significantly different from 
nontransfected cells or cells expressing PTPL1 alone. These 
results confirm a role for PTPL1 in modulating cell death 

Tx’d: BFP-TRPM2 + + +
GFP-PTPL1 + +
Anti-PTPL1 + kDa
WB: Anti-TRPM2-C
Anti-tubulin

Fig. 7. Expression of BFP-TRPM2 and PTPL1 in HEK-293T cells transfected for DVL. Lysates were prepared from HEK-293T cells transfected (Tx’d) with BFP-TRPM2 in pQB50, PTPL1 in pTracer-CMV or empty pTracer-CMV vector. One hundred micrograms of protein was loaded in each lane. Western 
blot (WB) analysis was performed with anti-PTPL1, anti-TRPM2, and anti- 
tubulin antibodies, followed by ECL. Two experiments were performed with 
similar results.
endogenous PTPL1 is involved in regulation of TRPM2 phosphorylation.

To determine the effect of downregulation of endogenous PTPL1 on the increase in [Ca\(^{2+}\)], in TRPM2-expressing cells, HEK-293T cells were transfected with BFP-TRPM2 alone or with control siRNA or siRNA targeted to PTPL1. [Ca\(^{2+}\)] was measured after treatment with vehicle (PBS) or 1 mM H\(_2\)O\(_2\). Cells expressing BFP-TRPM2 alone or BFP-TRPM2 and control siRNA demonstrated a similar rise in [Ca\(^{2+}\)], after treatment with H\(_2\)O\(_2\) (296 ± 14%, 281 ± 14% increase above baseline, respectively; see Table 2). Cells transfected with BFP-TRPM2 and siRNA targeted to PTPL1 demonstrated a significantly greater increase in [Ca\(^{2+}\)], after treatment with H\(_2\)O\(_2\) (441 ± 23% increase above baseline) compared with
cells expressing BFP-TRPM2 alone or BFP-TRPM2 with control siRNA (Table 2; \( P \leq 0.0001 \)). These results suggest that downregulation of the phosphatase PTPL1 is accompanied by enhanced TRPM2 channel activation by H$_2$O$_2$.

To assess the role of endogenous PTPL1 in modulating susceptibility to cell death through TRPM2, HEK-293T cells were transfected with empty pcDNA3.1/V5-His TOPO vector, TRPM2, and either control siRNA or siRNA targeted to PTPL1. Western blot analysis of lysates was performed, and blots were probed with anti-PTPL1, anti-V5-HRP, or anti-actin antibodies, followed by ECL. Representative results of four experiments are shown. B: effect of depletion of endogenous PTPL1 on TRPM2 tyrosine phosphorylation. HEK-293T cells transfected with V5-TRPM2 and either control siRNA or siRNA targeted to PTPL1 were treated with H$_2$O$_2$ for 0–20 min. Lysates were prepared at 0, 5, 10, 15, and 20 min, and immunoprecipitation performed with anti-V5 antibody. Western blots were probed with anti-phosphotyrosine and anti-V5 antibodies, followed by ECL. A representative result of four experiments is shown. C: quantification of results with densitometry. Densitometry measurements were performed on tyrosine phosphorylated TRPM2 bands and normalized by comparison to total V5-TRPM2. The means ± SE %increase in the intensity of the TRPM2 tyrosine phosphorylated band/total TRPM2 above baseline from four experiments is shown. 100% = time 0 phosphorylation in cells transfected with V5-TRPM2 and control siRNA.
with control siRNA was similar. In contrast, viability of cells expressing TRPM2 and siRNA targeted to PTPL1 was significantly worse than cells transfected with TRPM2 alone or TRPM2 with control siRNA (P ≤ 0.02) (Fig. 10A). All four experiments showed similar results.

To examine the role of endogenous PTPL1 in modulating susceptibility to apoptosis or necrosis in TRPM2-expressing cells with another approach, HEK-293T cells expressing V5-TRPM2 and siRNA targeted to PTPL1 or control siRNA were treated with 1 mM H2O2. Apoptosis was assessed at 6 h after treatment by labeling cells with Alexa Fluor 488 annexin V conjugates, and necrosis was assessed by labeling with PI, followed by fluorescence microscopy. Representative results are shown in Fig. 10B. Treatment of HEK-293T cells transfected with TRPM2 and siRNA targeted to PTPL1 with 1 mM H2O2 resulted in significantly more cells stained with annexin V (23 ± 3%, 1,303 cells counted) or PI (22 ± 3%) than cells transfected with TRPM2 and control siRNA (14 ± 1% labeled with annexin V, 13 ± 2% labeled with PI, 1,241 cells counted, P ≤ 0.02). These experiments support the conclusion that reduction in PTPL1 expression results in increased cell death through both apoptotic and necrotic processes in TRPM2-expressing cells.

**DISCUSSION**

TRPM2 is a calcium-permeable channel that is widely recognized to play a role in the susceptibility of cells to death in response to oxidative stress, TNFα, amyloid β-peptide, and concanavalin A (14, 16, 17, 55). PTPL1 is a tyrosine phosphatase that plays an important role in resistance to apoptosis and tumorigenesis (2, 23, 53). Here, we demonstrate that TRPM2 is tyrosine phosphorylated following treatment with H2O2 or TNFα and that TRPM2 is a target for dephosphorylation and inactivation by PTPL1. Modulation of TRPM2 function by PTPL1 may be a novel mechanism that contributes to the ability of PTPL1 to mediate resistance to apoptosis and death in some cell types.

The first finding of this report is that TRPM2 is tyrosine phosphorylated in response to H2O2 or TNFα. Other TRP channels that are tyrosine phosphorylated in response to agonists include TRPC3 (47), TRPC6 (21), TRPM7 (24), and TRPV4 (52). The ability of the broad tyrosine kinase inhibitor genistein and the Src kinase family inhibitor PP2 to block the rise in [Ca2+]i in TRPM2-expressing cells exposed to H2O2 or TNFα suggests that a Src kinase is responsible for TRPM2

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Table 2. [Ca2+]i response of HEK-293T cells expressing TRPM2 and siRNA targeted to PTPL1

Values are means ± SE; n, no. of cells studied. siRNA, small interfering RNA. Fura red-loaded HEK-293T cells were transfected with BFP-TRPM2 alone, or with control siRNA or siRNA targeted to PTPL1. Cells were treated with PBS or 1 mM H2O2 for 0–20 min. [Ca2+]i was measured at baseline, and the peak measurement was obtained during monitoring at 1- to 2- min intervals for 20 min after treatment. %Increase above baseline represents peak [Ca2+]i, divided by baseline × 100% − 100% (baseline). *P ≤ 0.02, significant difference in %increase from cells transfected with BFP-TRPM2 and treated with H2O2.
tyrosine phosphorylation. The identity of this kinase and the critical TRPM2 tyrosines that are phosphorylated are currently being investigated. Of note, other known targets for dephosphorylation by PTPL1 are also phosphorylated by Src kinases (23, 49).

The second major finding of this report is that the phosphatase PTPL1 associates with TRPM2. The interaction of PTPL1 with TRPM2 was demonstrated by three different approaches: using a TransSignal PDZ Domain Array blot, immunoprecipitation, and GST-pulldown assays with each of the five PDZ domains of PTPL1. TRPM2 was shown by both TransSignal PDZ Domain Array blots and GST-pulldown assays to interact with the first and fifth PDZ domains of PTPL1. Previously, PDZ1 of PTPL1 was shown to interact with the bromodomain-containing protein BP75 and the transcription regulator IkBα (13). TRPM2 is the first protein demonstrated to interact with the PDZ5 domain of PTPL1. The functional importance of the association of TRPM2 with these two PDZ domains of PTPL1 is not clear. These interactions may have a role in complex formation with other proteins that associate with the PDZ domains of PTPL1, or may simply function to bring TRPM2 into close proximity with the phosphatase domain of PTPL1. In addition, the PDZ5 domain and PDZ2 and PDZ3 of PTPL1 have been shown to interact with 1-phosphatidylinositol 4,5-biphosphate (13). Interaction with 1-phosphatidylinositol 4,5-biphosphate may be important for the membrane localization of PTPL1, bringing it into proximity with TRPM2 at the plasma membrane surface and resulting in the interactions which modulate TRPM2 activation.

The third major finding in this report is that TRPM2 phosphorylation is functionally important. Using two different kinase inhibitors, we demonstrated that inhibition of TRPM2 phosphorylation observed in response to oxidative stress blocked the rise in [Ca\(^{2+}\)], in cells expressing TRPM2. However, because kinase inhibitors lack specificity, we examined the functional role of tyrosine phosphorylation of TRPM2 using two other approaches. First, we overexpressed the tyrosine phosphatase PTPL1, demonstrating a decrease in TRPM2 tyrosine phosphorylation, reduction in the expected rise in [Ca\(^{2+}\)], and preservation of cell viability following treatment with H\(_2\)O\(_2\) or TNFα. Using a second, more physiologically relevant approach, we downregulated endogenous PTPL1, and demonstrated an increase in TRPM2 tyrosine phosphorylation, a significantly greater rise in [Ca\(^{2+}\)], and reduced cell viability in TRPM2-expressing cells treated with H\(_2\)O\(_2\) or TNFα. Together, these results support the conclusion that tyrosine phosphorylation of TRPM2 is involved in its regulation and that TRPM2 phosphorylation and activation are modulated by PTPL1 expression.

The mechanisms through which tyrosine phosphorylation of TRPM2 influences its activation are not known, but potential pathways include phosphorylation of tyrosines in the TRPM2 NUDT9-H domain, which may affect the efficiency of ADP ribose binding (28); phosphorylation of calmodulin binding sites, which may affect the efficiency of calmodulin interaction with TRPM2, which provides positive feedback for channel activation following Ca\(^{2+}\) influx (44); or phosphorylation of sites influencing the tertiary structure of TRPM2, enhancing pore opening. To identify the mechanisms, we are currently in the process of identifying specific tyrosines on TRPM2, which are phosphorylated in response to oxidative stress.

The level of PTPL1 expression correlates positively with resistance to apoptosis, and mechanisms through which PTPL1 may mediate its effects on apoptosis include regulation of Fas cell surface expression (23) or through the phosphatidylinositol 3-kinase pathway (6, 26). Here, we provide evidence that TRPM2, which mediates susceptibility to cell death in response to oxidative stress, TNFα, and amyloid β-peptide (14, 17, 55), is another target of PTPL1 through which resistance to apoptosis and cell death may be mediated. We hypothesize that association of endogenous TRPM2 with PTPL1 has a role in maintaining TRPM2 in a dephosphorylated and inactive state. Under oxidative stress and other conditions which enhance TRPM2 tyrosine phosphorylation, the association of TRPM2 and PTPL1 contributes to dephosphorylation of activated TRPM2 and containment of Ca\(^{2+}\) influx, preserving cell viability. Although Hara et al. (17) suggested that cell death in TRPM2-expressing cells treated with 100–300 μM H\(_2\)O\(_2\) was not associated with activation of caspase 3 or DNA laddering and therefore was primarily necrotic, we observed an increase in both apoptotic and necrotic death, consistent with previous findings demonstrating caspase cleavage in TRPM2-expressing cells after H\(_2\)O\(_2\) treatment (55). These differences may reflect different H\(_2\)O\(_2\) concentrations, cells lines, or other experimental conditions. The role of TRPM2 in concanavalin A-mediated inward current and concanavalin A-induced death of Jurkat cells was recognized recently (16). As additional agonists that activate TRPM2 are identified, understanding the mechanisms that regulate TRPM2 activation and its modulation of cell death is becoming increasingly important.

ACKNOWLEDGMENTS

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GRANTS

This work was supported by National Institutes of Health Grants R01 DK-46778, R01 HL-58672, and R01 HL-74854, and by the Four Diamonds Fund of the Pennsylvania State University College of Medicine.

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