TRPM2 is a Ca\[^{2+}\]\,-permeable channel that is activated by oxidative stress and confers susceptibility to cell death. Here, an isoform of TRPM2 was identified in normal human bone marrow that consists of the TRPM2 N terminus and the first two predicted transmembrane domains. Because of alternative splicing, a stop codon (TAG) is located at the splice junction between exons 16 and 17, resulting in deletion of the four C-terminal transmembrane domains, the putative calcium-permeable pore region, and the entire C terminus. This splice variant was found in other hematopoietic cells including human burst forming unit-erythroid-derived erythroblasts and TF-1 erythroleukemia cells. Endogenous expression of both the short form of TRPM2 (TRPM2-S) and the full length (TRPM2-L) was determined by reverse transcriptase-PCR, and localization of endogenous TRPM2 to the plasma membrane was demonstrated by confocal microscopy. Heterologous expression of TRPM2-S in HEK 293T cells demonstrated similar membrane localization as TRPM2-L, and coexpression of TRPM2-S did not alter the subcellular localization of TRPM2-L. The direct interaction of TRPM2-S with TRPM2-L was demonstrated with immunoprecipitation. H\(_2\)O\(_2\) induced calcium influx through TRPM2-L expressed in 293T cells. Coexpression of TRPM2-S suppressed H\(_2\)O\(_2\)-induced calcium influx through TRPM2-L. Furthermore, expression of TRPM2-S inhibited susceptibility to cell death and onset of apoptosis induced by H\(_2\)O\(_2\) in cells expressing TRPM2-L. These data demonstrate that TRPM2-S is an important physiologic isoform of TRPM2 and modulates channel activity and induction of cell death by oxidative stress through TRPM2-L.

Regulation of the intracellular calcium concentration [Ca\(^{2+}\)]\(_i\), is of critical importance in determination of cell fate. In many cell types, oxidative stress, through the production of oxygen metabolites including H\(_2\)O\(_2\), causes an increase in [Ca\(^{2+}\)]\(_i\), which results in cell injury, apoptosis, or necrosis (1, 2). One mechanism through which H\(_2\)O\(_2\) may disrupt calcium homeostasis has recently been identified. A widely expressed Ca\(^{2+}\)-permeable cation channel, TRPM2, can be activated by molar levels of H\(_2\)O\(_2\) and other agents that produce reactive oxygen species (3, 4). This channel is part of a physiological pathway through which H\(_2\)O\(_2\) and tumor necrosis factor \(\alpha\) may induce cell death (3).

TRPM2, also called LTRPC-2 or TRPC7, is a member of the transient receptor potential (TRP\(^1\)) protein superfamily. This is a diverse group of calcium-permeable cation channels expressed on nonexcitable cells, related to the archetypal TRP, Drosophila Trp (5–7). The TRP superfamily, conserved from Caenorhabditis elegans to humans, has been divided into six subfamilies. Mammalian isoforms share six putative transmembrane domains similar to the core structure of many pore-forming subunits of voltage-gated channels except that they lack positively charged residues necessary for the voltage sensor. One subfamily of TRP channels are referred to as TRPMs (3, 4, 8–14), because the first described member was melastatin (MLSN), a putative tumor suppressor protein (6, 7). Prior to implementation of a unified nomenclature for the TRP superfamily, this subfamily was also known as LTRPC, named because of longer open reading frames of ~1600 amino acids (5). Although the mechanisms of activation of specific TRPM are not known, some TRPM appear to have important roles in cell proliferation. For example, TRPM1 (MLSN) is expressed in melanocytes, and its level of expression correlates inversely with melanoma aggressiveness and the potential for melanoma metastasis (8, 9). TRPM5 (MTR1 and LTRPC5) is located in the Beckwith-Wiedemann syndrome critical region of human chromosome 11, although its function in cell growth is not known (10). TRPM8 (Trp-p8) has significant homology to human melastatin, but it is up-regulated in prostate cancer and a number of nonprostatic neoplastic tumors (13).

TRPM2 has been cloned from human brain, lymphocytes, and monocytes (11, 16, 17). TRPM2 is activated by H\(_2\)O\(_2\) and other agents that produce reactive oxygen species, resulting in an increase in the intracellular free calcium concentration ([Ca\(^{2+}\)]\(_i\)) (3, 4). Heterologous expression of TRPM2 in 293 cells conferred susceptibility to H\(_2\)O\(_2\)-induced cell death, which correlated with the elevation in [Ca\(^{2+}\)]\(_i\). Furthermore, suppression of endogenous TRPM2 expression in rat insulinoma RIN-5F or monocyte U937 cells resulted in significantly diminished Ca\(^{2+}\) influx and cell death induced by H\(_2\)O\(_2\) or tumor necrosis factor \(\alpha\) (3). These data strongly support the physiologic role of TRPM2 as an endogenous H\(_2\)O\(_2\)-activated calcium-permeable channel that mediates cell death following oxidative stress.

In this report, a truncated isoform of TRPM2 was identified.
in human hematopoietic cells that lacks four of the six predicted C-terminal transmembrane domains and the putative pore region permeable to calcium. The short form of TRPM2 (TRPM2-S) was determined to interact directly with full-length (TRPM2-L). Using a digital video imaging system in which single 293T cells that express transfected TRPM2-S were identified by detection of green fluorescent protein (GFP), cells that expressed TRPM2-L were identified by detection of blue fluorescent protein (BFP), and [Ca^{2+}]_i, was simultaneously measured by Fura Red fluorescence, the ability of TRPM2-S to suppress H_2O_2-induced calcium influx through TRPM2-L was demonstrated. In addition, the short form of TRPM2 inhibited susceptibility to cell death induced by H_2O_2 through full-length TRPM2. These data suggest that the interaction between TRPM2-S and TRPM2-L is an important mechanism for regulating channel activity as well as the cellular response to oxidative stress.

### EXPERIMENTAL PROCEDURES

#### Culture of Cell Lines and Human BFU-E-derived Cells—Jurkat cells, K562, AML-193, and TF-1 cells were obtained from the American Type Culture Collection (Manassas, VA). Jurkat cells were cultured in RPMI 1640 with 10% fetal bovine serum (FBS), K562 cells were cultured in Iscove's modified Dulbecco's medium with 10% FBS, AML-193 cells were cultured in Iscove's modified Dulbecco's medium with 0.005 mg/ml insulin, 0.005 mg/ml transferrin, 5 ng/ml granulocyte-macrophage colony-stimulating factor, and 5% FBS. TF-1 cells were cultured in RPMI 1640 medium with 1 ng/ml granulocyte-macrophage colony-stimulating factor. HEK 293 cells were obtained from the American Type Culture Collection, and 293T cells were obtained from Dr. Dwayne Barber (Ontario Cancer Institute, Toronto, Canada). Both were cultured in Dulbecco's modified Eagle's medium with 10% FBS. Peripheral blood from volunteer donors was obtained under protocols approved by the Geisinger Institutional Review Board. Human BFU-E were cultured in methyl cellulose medium, and BFU-E-derived cells were harvested at day 10 as described previously (18).

#### RT-PCR of TRPM2 in Human Primary Cells and Cell Lines—RNA was prepared from human 293T, Jurkat, K562, AML-193, TF-1 cells, and BFU-E-derived cells. cDNA was prepared from RNA using the SuperScript First Strand Synthesis System (Invitrogen) for RT-PCR. RT-PCR was performed for 35 cycles (denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s, and extension at 72 °C for 30 s). Primers for TRPM2 were 5' primer (5'-TCGGGCCCCAACCACACAGCTGA-3') and 3' primer (5'-CGCATGCTGCTGGTTCAAGATG-3'). Control 18 S rRNA primers used in RT-PCR were 5' primer (5'-GAAAGTCGGGAGGTTCGGTG-3') and 3' primer (5'-CGTCATTCTGGTCCTGGAAGTG-3'). Control 18 S rRNA primers used in RT-PCR were 5' primer (5'-GAAAGTCGGGAGGTTCGGTG-3') and 3' primer (5'-CGTCATTCTGGTCCTGGAAGTG-3'). Control 18 S rRNA primers used in RT-PCR were 5' primer (5'-GAAAGTCGGGAGGTTCGGTG-3') and 3' primer (5'-CGTCATTCTGGTCCTGGAAGTG-3').

#### Cloning of TRPM2-L and TRPM2-S—TRPM2-L and TRPM2-S were cloned from human bone marrow Marathony-Reddy cDNA (Clontech, Palo Alto, CA) by amplifying five adjacent cDNA fragments encoding the complete open reading frame of TRPM2 with five PCR reactions. The primers were chosen based on the published cDNA sequence of TRPM2 (GenBank™ accession number AB001535). For the first fragment, the primers were chosen to the region between nucleotides 361 and 1572 (1447-nucleotide fragment). The nucleotide sequences of the forward and reverse primers are: forward primer (5'-GAAAGT CGGAGGTTCG-3'), reverse primer (5'-GAAAGT CGGAGGTTCG-3'). PCR was used to amplify the third fragment between nucleotides 2261 and 3376 using the 5' primer (5'-TCCCTCTACACGGCTTCCCTCAG-3') and the 3' primer (5'-GTTGAGGTAGAAGTGGTAGACG-3'). The PCR products were cloned into the TA vector, and the insert was sequenced to confirm the presence or absence of the TAG stop codon.

#### Generation of Antibodies Specific to TRPM2—Two rabbit polyclonal antibodies were generated to TRPM2 and affinity-purified by Bethyl Laboratories (Montgomery, TX). One of these antibodies (anti-TRPM2-N) was generated to an epitope in the N terminus of TRPM2 (ILKELSKEEDTDSSEMLA, amino acids 659–677) and recognized both full-length (TRPM2-L) and TRPM2-S. A second antibody (anti-TRPM2-C) was generated to an epitope in the C terminus of TRPM2 (KAAEEDAEPEG-GRKKTTEPGDS, amino acids 1216–1237) and was specific for TRPM2-L. The specificity of the antibodies was confirmed using in vitro translation products prepared with cDNAs for mTRPC2 clone 14 (19), hTRPC6 (20), TRPM2-S, and TRPM2-L, cloned into pcDNA3 or pcDNA3.1/V5-His TOPO vector as described previously (21). In vitro translation products were prepared with the TnT quick coupled transcription/translation system (Promega, Madison, WI).

#### Immunoblotting of Whole Cell Lysates and Crude Membrane Preparations—Human 293T cells were transiently transfected using LipofectAMINE Plus Reagent with vector alone, TRPM2-S, TRPM2-L, or a combination of TRPM2-S and TRPM2-L. The cells were studied 48 h after transfection. For Western blotting of whole cell lysates, the lysates were separated on 8% polyacrylamide gels, followed by transfer to polyvinylidene difluoride membranes. Following blocking, the blots were incubated with primary antibody (anti-TRPM2-N, 1:300; anti-TRPM2-C, 1:1500; anti-V5, 1:2000 (Invitrogen); anti-TRPC6, 1:200 (Alomone Laboratories, Jerusalem, Israel)), washed, and then incubated with horseradish peroxidase-conjugated anti-rabbit or antimouse antibody (1:2000). ECL was used for detection of signal. The crude membrane preparations were also prepared from 293T cell pellets transfected with vector alone (pcDNA3.1), TRPM2-S, and/or TRPM2-L as described previously (21), and Western blotting was performed as described above.

#### Immunoprecipitation—To determine whether anti-TRPM2-N and anti-TRPM2-C antibodies are able to immunoprecipitate their targets, TRPM2-S and TRPM2-L proteins were prepared from TRPM2-S and TRPM2-L cDNAs subcloned into pET-42b and expressed with the TNT transcription/translation system (Promega, Madison, WI). The cells were routinely studied 48 h after transfection. For Western blotting of whole cell lysates, the lysates were separated on 8% polyacrylamide gels, followed by transfer to polyvinylidene difluoride membranes. Following blocking, the blots were incubated with primary antibody (anti-TRPM2-N, 1:300; anti-TRPM2-C, 1:1500; anti-V5, 1:2000 (Invitrogen); anti-TRPC6, 1:200 (Alomone Laboratories, Jerusalem, Israel)), washed, and then incubated with horseradish peroxidase-conjugated anti-rabbit or antimouse antibody (1:2000). ECL was used for detection of signal. The crude membrane preparations were also prepared from 293T cell pellets transfected with vector alone (pcDNA3.1), TRPM2-S, and/or TRPM2-L as described previously (21), and Western blotting was performed as described above.

#### Immunoprecipitation of TRPM2 in TF-1 Erythroid Cell Lines and in TF-1 Transfected 293T Cells—TRPM2-L and TRPM2-S were transfected into each well of Lab-Tek Permanox Chamber Slides precoated with fibronectin. After 30 min, the cells were washed three times with phosphate-buffered saline, fixed with methanol at −20 °C for 10 min, and permeabilized in 0.5% Triton X-100 in phosphate-buffered saline for 5 min. Incubation for 10 min in
29% goat serum preceded staining with primary antibody (anti-TRPM2-N or anti-TRPM2-C, 1:50) for 20 min at room temperature, followed by secondary antibody (goat anti-rabbit Alexa 488; Molecular Probes, Eugene, OR) for 20 min in the dark. The slides were stained with propidium iodide (PI) in Vectashield mounting medium (Vector Laboratories, Burlingame, CA). The images were acquired with a Leica TCS SP2 Confocal Microscope.

293T cells transfected with TRPM2-L in pcDNA3.1, TRPM2-S in pcDNA3.1/V5-His TOPO, or both were plated on polylysine-coated chamber slides 24 h after transfection and incubated for 24 h more at 37 °C. The cells were fixed, permeabilized, and incubated with primary (rabbit anti-TRPM2-C or mouse anti-V5) or secondary antibodies (fluorescein isothiocyanate donkey anti-rabbit IgG, Jackson Laboratories, West Grove, PA; Texas Red goat anti-mouse) as described above. The slides were stained with DAPI in Vectashield mounting medium to visualize DNA instead of PI, which could not be distinguished from Texas Red fluorescence. To visualize DAPI, the cells were viewed using a Nikon Operetta-2 microscope equipped for epifluorescence. The images were acquired with an air-cooled CCD SenSys digital camera from Photometrics (Tucson, AZ) and processed using IPLab and Enhanced Photon Reassignment software programs obtained from Scanalytics (Fairfax, VA).

Measurement of [Ca2+]i, with Digital Video Imaging—293T cells were transfected with TRPM2-S subcloned into pTracer-CMV, TRPM2-L subcloned into pQBI50, or both as described above. The pTracer-CMV vector contains an SV40 promoter driving expression of a GFP gene and a CMV promoter driving expression of TRPM2-S. The pQBI50 vector contains a CMV promoter that drives expression of SuperGlo BFP fused through a flexible linker to TRPM2-L. Successful transfection of 293T cells with TRPM2-S and TRPM2-L was verified by detection of GFP (excitation, 478 nm; emission, 535 nm) and BFP (excitation, 380 nm; emission 435 nm), respectively, in cells expressing the fluorescent indicator Fura Red (excitation, 460 and 490 nm; emission 435 nm), respectively, in cells with our digital video imaging system (21, 22). The fluorescence microscopy-coupled digital video imaging used to measure changes in [Ca2+]i has been described previously (22–25). To study changes in [Ca2+]i, in transfected cells, we were not able to use Fura-2 as the detection fluorophore because its excitation and emission wavelengths overlap with those of GFP. Instead, we used the fluorescent sensor indicator Fura Red (excitation, 460 and 490 nm; emission, 500-nm-long pass), a dual wavelength excitation probe. We determined $R_{\text{min}}$ (minimum fluorescence intensity ratio (r) of the emission following excitation at 460 nm divided by the emission following excitation at 490 nm) and $R_{\text{max}}$ (maximum r) and the constants $K_{D/R}$ with Fura Red so that $[\text{Ca}^{2+}]$, could be calculated using the formula $[\text{Ca}^{2+}] = -K_{D/R} (1/R_{\text{min}} - 1/R_{\text{max}} - 1)(S_{\text{Fura Red}})_{\text{max}}$.

Transfected 293T cells grown on glass coverslips were loaded at 48 h with 5 μM Fura Red-AM for 30 min at 37 °C in the presence of Pluronic F-127 to enhance loading. Transfected 293T cells were treated with 0–10 mM H2O2 and [Ca2+]i was measured at base line and over a 20-min interval. Statistical significance of results was analyzed with one-way analysis of variance. Assays of Cell Viability—293T cells transfected for 48 h with vector, TRPM2-S, or TRPM2-L, or both in pQBI50 were treated with 0–10 mM H2O2 for 40 min. Cell viability was assessed by trypan blue exclusion. Induction of apoptosis or necrosis was also assessed in these cells following treatment with H2O2 with the Vibriant apoptosis assay kit 2 (Molecular Probes), in which apoptotic cells are labeled with annexin V conjugated to Alexa Fluor 488 and necrotic cells are labeled with propidium iodide.

RESULTS

Cloning of TRPM2-S—TRPM2 has been reported to be highly expressed in lymphocytes as well as in granulocytes and other hematopoietic cell lines (4, 17). To study TRPM2 in human hematopoietic cells, the expression of TRPM2 in different hematopoietic lineages was first examined. RT-PCR was performed on RNA isolated from Jurkat (T cell lymphoblast cell line), K562 (chronic myelogenous leukemia cell line), AML-193 (acute monocytic leukemia cell line), and TF-1 (erythroleukemia cell line) and from BFU-E-derived primary erythroid cells (18). The results are shown in Fig. 1. TRPM2 mRNA was highly expressed in Jurkat, K562, AML-193, and TF-1 cells. TRPM2 was also expressed, although at lower levels, in primary human erythroblasts at day 10 of culture, which are largely proerythroblasts (18). TRPM2 mRNA was detectable at low levels in 293 cells; these results differ from a previous report in which TRPM2 mRNA was undetectable in 293 cells (17), possibly because of differences in the RT-PCR conditions or in the 293 cells studied. No TRPM2 bands were observed when PCR was performed without the reverse transcriptase step, demonstrating that these products did not result from contaminating DNA. The identity of PCR bands was confirmed by sequencing.

To clone TRPM2 for further study, a strategy was designed based on the published cDNA sequence (see “Experimental Procedures”). Using Marathon-Ready cDNA from human bone marrow as the template, five adjacent cDNA fragments encoding the complete open reading frame of TRPM2 were amplified by PCR. Following sequencing, the fragments were ligated together to produce full-length TRPM2 (TRPM2-L). Sequencing of the third fragment resulted in two alternative sequences; one was identical to the published sequence, and the other included a TAG stop codon at nucleotides 2984–2986. This sequence is a result of alternative splicing of the 3’ end of the intron between exons 16 and 17. As shown on Fig. 2, this isoform, TRPM2-S, results in the deletion of the entire C terminus of full-length TRPM2, including the four C-terminal transmembrane domains and the putative pore region permeable to calcium. Because the TAG stop codon immediately follows the CAG encoding glutamine (Fig. 2), no unique amino acids are introduced in the TRPM2-S protein.

The PCR products following amplification of bone marrow cDNA with primers for the third fragment of TRPM2 were subcloned into the T/A vector, and 13 clones were sequenced. The sequences of seven different clones were identical to TRPM2-S, and six were identical to TRPM2-L. To determine whether TRPM2-S is expressed in other hematopoietic cells, cDNA was also prepared with RNA from normal human BFU-E-derived cells and from TF-1 cells. As described above, PCR products amplified with primers for the third fragment were subcloned into the T/A vector, and the clones were sequenced. Four of eight clones from normal human BFU-E derived cells, and two of three clones from TF-1 cells were identical to TRPM2-S; the rest were identical to TRPM2-L. These data demonstrate the presence of TRPM2-S in three different hematopoietic cell types.

Generation of Antibodies Specific to TRPM2—To study the interaction and function of TRPM2 isoforms, an affinity-purified antibody was generated to the N terminus of TRPM2 (anti-TRPM2-N), which recognizes both TRPM2-S and TRPM2-L. A second antibody was generated to the C terminus of TRPM2 (anti-TRPM2-C), which recognizes only TRPM2-L. To characterize antibody specificity, in vitro translation was performed using cDNAs for TRPM2-S and TRPM2-L in pcDNA3.1/V5-His TOPO and mTRPC2 clone 14 and hTRPC6 in pcDNA3 as controls. Western blotting was performed with each of these in vitro translation products with anti-TRPM2-N, anti-TRPM2-C, anti-V5, or anti-TRPC6 antibodies. The results are shown in Fig. 3A. Anti-TRPM2-N recognized the in vitro translation products TRPM2-S (95
Fig. 2. Schema of cDNA for the two splice variants TRPM2-L and TRPM2-S. The protein structures of TRPM2-L and TRPM2-S are shown with the predicted transmembrane domains and the pore region indicated. Nucleotide and amino acid sequences surrounding the alternatively spliced stop codon between exons 16 and 17 are shown below the TRPM2 gene, demonstrating termination of TRPM2 after the second transmembrane domain in TRPM2-S.

Fig. 3. A, specificity of TRPM2 antibodies. In vitro translation products were prepared using cDNAs of mTRPC2 clone 14 (pcDNA3), hTRPC6 (pcDNA3), and TRPM2-S and TRPM2-L in pcDNA3.1/V5-His TOPO. Equivalent amounts of each reaction were loaded in each lane, and Western blotting was performed. The blots were probed with anti-TRPM2-N, anti-V5, anti-TRPM2-C, and anti-TRPC6 antibodies (A). B, 293T cells transfected with TRPM2-S were stained with anti-V5 as the primary antibody and Texas Red anti-mouse IgG as the secondary antibody. DAPI was used to stain DNA. C, Western blot of transfected 293T cells. The lysates were prepared from 293T cells transfected with both TRPM2-S and TRPM2-L, were stained with anti-V5 and goat anti-mouse secondary antibodies (F) and anti-TRPM2-C and donkey anti-rabbit secondary antibodies (G). The merged images of F and G are shown in H and demonstrate significant overlap.

Expression of Endogenous TRPM2—To confirm the expression and determine the subcellular localization of endogenous TRPM2, immunolocalization studies were performed with TF-1 cells using anti-TRPM2-N and anti-TRPM2-C antibodies and confocal microscopy. PI staining was used to localize DNA. Representative results shown here demonstrate that endogenous TRPM2 recognized by either anti-TRPM2-N (Fig. 4A) or anti-TRPM2-C (Fig. 4B) is localized on or near the plasma membrane. In Fig. 4C, no fluorescence was observed in control cells stained with secondary antibody alone. Unfortunately, anti-TRPM2-N antibodies cannot distinguish TRPM2-S and TRPM2-L subcellular localization of TRPM2-S in transfected cells. These observations were confirmed with antibody directed to the V5 tag. Fig. 3B also demonstrates the inability to detect endogenous TRPM2 protein expression in 293T cells under the identical conditions, suggesting that endogenous protein levels are low or absent.

Subcellular Localization of TRPM2-S and TRPM2-L—To distinguish the subcellular localization of TRPM2-S or TRPM2-L isoforms and determine whether TRPM2-S alters the localization of TRPM2-L (10), 293T cells were transfected with TRPM2-S in pcDNA3.1/V5-His TOPO, TRPM2-L in pcDNA3.1, or both. TRPM2-S was detected with antibody to the V5 tag, and TRPM2-L was detected with antibody specific for the C terminus of TRPM2. Cell staining was visualized by fluorescence microscopy. The images at different planes through the cell were deconvolved (Scanalytics software) to remove out-of-focus contaminating light to generate high reso-
The expression pattern was not different from that observed previously (21). Western blotting was performed with precipitated proteins (P) or supernatant before the first wash (S). The proteins were detected by horseradish peroxidase-streptavidin followed by ECL. B, 293T cells were transfected with vector alone (pcDNA3.1), TRPM2-S in pcDNA3.1/V5-His TOPO, TRPM2-L in pcDNA3.1, or both. The cell lysates were immunoprecipitated with anti-TRPM2-C or anti-V5 antibodies. Western blotting was performed with anti-TRPM2-C antibody, and the blots were then stripped and reprobed with anti-V5 antibody.

To confirm that TRPM2-S and TRPM2-L have a similar subcellular distribution and that TRPM2-S does not alter the subcellular localization of TRPM2-L, crude membrane fractions were prepared from 293T cells transfected with vector alone (pcDNA3.1), TRPM2-S, TRPM2-L, or both as described previously (21). Western blotting was performed with protein isolated in the crude membrane pellet or the supernatant, and the blots were probed with anti-TRPM2-N (A) or anti-TRPM2-C antibodies (B).

When TRPM2-S and TRPM2-L were coexpressed in the same 293T cells (Fig. 4, E–H), the expression pattern was not different from that observed when each was expressed alone, and the spatial distribution showed extensive overlap. The results were identical when a range of DNA concentrations was used to transfect 293T cells to reduce the level of expressed protein. The results were also similar when CHO, CHO-S, or COS-1 cells were transfected (not shown). When anti-V5, anti-TRPM2-N, or anti-TRPM2-C antibodies were used with the appropriate secondary antibody to stain 293T cells transfected with vector alone, no positive fluorescence was observed (not shown). These data show that transfected TRPM2-L and TRPM2-S are also expressed at or near the plasma membrane.

To determine whether TRPM2-S directly interacts with TRPM2-L, 293T cells were transfected with empty vector, TRPM2-S in pcDNA3.1/V5-His TOPO, TRPM2-L in pcDNA3.1, or both. Each cell lysate was immunoprecipitated with anti-TRPM2-C to precipitate TRPM2-L or anti-V5 to precipitate TRPM2-S. The blots were probed with anti-TRPM2-C or anti-TRPM2-N antibodies, and Western blotting was performed with precipitated proteins (P) or supernatant before the first wash (S). The proteins were detected by horseradish peroxidase-streptavidin followed by ECL. B, 293T cells were transfected with vector alone (pcDNA3.1), TRPM2-S in pcDNA3.1/V5-His TOPO, TRPM2-L in pcDNA3.1, or both. The cell lysates were immunoprecipitated with anti-TRPM2-C or anti-V5 antibodies. Western blotting was performed with anti-TRPM2-C antibody, and the blots were then stripped and reprobed with anti-V5 antibody.

To study the interaction of TRPM2 isoforms with each other, the ability of anti-TRPM2 antibodies to immunoprecipitate their targets was first examined. TRPM2-S and TRPM2-L cDNAs in pET-42b were expressed by in vitro translation and labeled using Biotin-Lysyl-tRNA. Each of these in vitro translation products was incubated with anti-TRPM2-C or anti-TRPM2-N antibodies and bound protein precipitated with protein A-Sepharose. Western blotting of pelleted fractions or the supernatants demonstrates that anti-TRPM2-C immunoprecipitates TRPM2-L but not TRPM2-S (Fig. 6A). The results also demonstrate that anti-TRPM2-N immunoprecipitates both TRPM2-S and TRPM2-L but that TRPM2-L is not immunoprecipitated as efficiently as TRPM2-S by this antibody. The higher molecular masses of proteins shown here compared with that in reticulocyte lysates or 293T cells transfected with TRPM2-S or TRPM2-L (Fig. 4, D–H) represents intracellular membrane structures in which TRPM2 is produced and transported.
A Novel TRPM2 Isoform Inhibits Calcium Influx and Cell Death

TRPM2-S Suppresses Susceptibility to Cell Death Induced through TRPM2 by H$_2$O$_2$—TRPM2-expressing cells are susceptible to cell death induced by exposure to H$_2$O$_2$ (3). Here, the ability of TRPM2-S to modulate cell death induced by H$_2$O$_2$ was examined. 293T cells were transfected with vector alone, TRPM2-S, TRPM2-L, or combinations in pQB50. Empty vector was transfected with TRPM2-S or TRPM2-L to maintain the equivalent amount of DNA used in cotransfection of both TRPM2-S and TRPM2-L. At 48 h, the cells were treated with 0, 0.1, 1.0, or 10 mM H$_2$O$_2$ for 40 min. The cell viability was then assessed by trypan blue exclusion. The asterisk indicates a significant difference from the other groups of transfected cells ($p < 0.01$). Mean trypan blue exclusion + S.E. for three experiments is shown. B. Western blot of 293T cells transfected with TRPM2 splice variants. 293T cells were transfected with equivalent amounts of vector alone (pQB50), TRPM2-S, TRPM2-L, or both. The cell lysates were prepared, and 100 µg of each was loaded in each lane. The blots were probed with anti-TRPM2-N or anti-TRPM2-C and secondary antibody followed by ECL.
A Novel TRPM2 Isoform Inhibits Calcium Influx and Cell Death

In this report, a new isoform of TRPM2, TRPM2-S, was cloned from human bone marrow and consists of only the N terminus and the first two transmembrane domains. Here, we demonstrated that expression of TRPM2-S inhibits calcium influx, enhances cell viability, and reduces apoptosis and cell death, which occur following exposure to full-length TRPM2-expressing cells to H$_2$O$_2$. Because H$_2$O$_2$ causes an increase in intracellular calcium that precedes cell death in numerous cell types including cardiac and smooth muscle cells, macrophages, and neurons (1), TRPM2-S may have a generally important role in the determination of cell fate following exposure to oxidative stress.

The first major finding of this report is the identification of a short isoform of TRPM2 expressed physiologically in human hematopoietic cells. This is the third TRPM family member for which a short isoform has been identified and is similar to the short isoforms of MLSN (TRPM1) (10) and MTR1 (TRPM5) (12) in that all have a deletion of the C terminus including transmembrane domains and the putative calcium-permeable pore region. N-terminal domains of TRPCs have previously been shown to bind to and suppress the activity of full-length TRPC proteins (26–28). These N-terminal domains of TRPCs were found to participate in heteromultimer complex formation. The physiological short form of MLSN, MLSN-S, which has no transmembrane domains, interacts with and suppresses the activity of full-length MLSN (MLSN-L) (10). MLSN-L localizes near or in the plasma membrane, whereas MLSN-S is uniformly distributed in the cytoplasm. Published data suggest that the suppression of activity by MLSN-S results from direct interaction of MLSN-S and MLSN-L, inhibiting translocation of MLSN-L to the plasma membrane (10). Here we identified a short isoform of TRPM2 that interacts with and suppresses the activity of full-length TRPM2, but the mechanism does not appear to be the same as reported for MLSN-S. Although TRPM2-S directly interacts with TRPM2-L, our experiments demonstrate that TRPM2-S and TRPM2-L have a similar subcellular localization and that TRPM2-S does not alter the localization of TRPM2-L. Unlike MLSN-S, TRPM2-S retains two transmembrane domains that localize it to the cell membrane.

The second major finding of this report is that TRPM2-S is able to inhibit calcium influx induced by H$_2$O$_2$ through full-length TRPM2. TRPM2 has been shown to be regulated by at least three different mechanisms. 1) TRPM2 has a Nudix box in its C terminus that has homology to NUDT9, an ADP-ribose pyrophosphatase degrading ADP-ribose (ADPR). Although it can function as an ADPR pyrophosphatase, it has a much lower level of activity than NUDT9 (11). The Nudix box may also serve as an ADPR-binding site, and ADPR has been shown to directly guilt TRPM2 opening (11, 17). Because in oxidative stress ADPR production is increased (29), this is a mechanism through which H$_2$O$_2$ may regulate TRPM2-L. 2) NAD has also been shown to directly activate TRPM2 (3, 17), although one group of investigators was unable to show that NAD stimulates current through TRPM2 (4). 3) H$_2$O$_2$, but not ADPR or NAD,
was able to stimulate calcium influx through an TRPM2 mutant with a small deletion in the C terminus (4). These data suggest that H$_2$O$_2$ and oxidative stress can mediate calcium influx through a third mechanism independently of ADPR or NAD. This pathway will need to be defined before the mechanism through which TRPM2-S inhibits H$_2$O$_2$-mediated calcium influx through TRPM2-L can be identified. Because TRPCs have been proposed to function as homo- or heterotetramers (30) and TRPM2-S and TRPM2-L are shown here to directly interact, TRPM2-L may participate in heterotetramer formation, altering the tertiary structure of the TRPM2-L homotetramer and inhibiting calcium permeability. Alternatively, because it is missing the C terminus, TRPM2-S could also function to impair localization of TRPM2-L to signaling complexes (6) or act as a dominant negative blocking an unknown aspect of TRPM2-L regulation critical for calcium channel activation.

The third and most important finding of this report is that TRPM2-S inhibits the decreased cell viability and increased susceptibility to cell death that result from activation of TRPM2-L by oxidative stress. Our observation that exposure of TRPM2-L-expressing cells to H$_2$O$_2$ reduces cell viability and enhances apoptosis is consistent with previous reports (3, 17). The significance of our work is the identification of a physiologic splice variant of TRPM2, which suppresses cell death in response to oxidative stress. The genomic sequence of TRPM2 spans 32 exons (16), raising the possibility that multiple splice variants with different capabilities may exist. In fact, two other splice variants of TRPM2 have been identified in granulocytes and undifferentiated HL-60 cells, which have small deletions in their N terminus (amino acids 538–557) or C terminus (amino acids 1292–1325) (4). H$_2$O$_2$ does not induce calcium influx in their N terminus (amino acids 538–557) or C terminus (amino acids 1292–1325) (4). H$_2$O$_2$ does not induce calcium influx in undifferentiated HL-60 cells, which have small deletions in their N terminus (amino acids 538–557) or C terminus (amino acids 1292–1325) (4). H$_2$O$_2$ does not induce calcium influx in

ability of TRPM2-S to inhibit H$_2$O$_2$-induced calcium influx through TRPM2-L and to suppress the susceptibility to cell death induced through TRPM2-L by H$_2$O$_2$. Our studies suggest that TRPM2-S may have an important role in many tissues because it can modulate calcium influx and cellular responses to oxidative stress.

REFERENCES
