OS-9 Regulates the Transit and Polyubiquitination of TRPV4 in the Endoplasmic Reticulum

Yan Wang, Xiao Fu, Stephanie Gaiser, Michael Köttgen, Albrecht Kramer-Zucker, Gerd Walz, and Tomasz Wegierski

From the Renal Division, University Hospital Freiburg, Hugstetter Strasse 55, Freiburg 79106, Germany

Transient receptor potential (TRP) proteins constitute a family of cation-permeable channels that are formed by homo- or heteromeric assembly of four subunits. Despite recent progress in the identification of protein domains required for the formation of tetramers, the mechanisms governing TRP channel assembly, and biogenesis in general, remain largely elusive. In particular, little is known about the involvement of regulatory proteins in these processes. Here we report that OS-9, a ubiquitously expressed endoplasmic reticulum (ER)-associated protein, interacts with the cytosolic N-terminal tail of TRPV4. Using a combination of co-expression and knockdown approaches we have found that OS-9 impedes the release of TRPV4 from the ER and reduces its amount at the plasma membrane. Consistent with these in vitro findings, OS-9 protected zebrafish embryos against the detrimental effects of TRPV4 expression in vivo. A detailed analysis of the underlying mechanisms revealed that OS-9 preferably binds TRPV4 monomers and other ER-localized, immature variants of TRPV4 and attenuates their polyubiquitination. Thus, OS-9 regulates the secretory transport of TRPV4 and appears to protect TRPV4 subunits from the precocious ubiquitination and ER-associated degradation. Our data suggest that OS-9 functions as an auxiliary protein for TRPV4 maturation.

Transient receptor potential (TRP) proteins constitute a family of cation-permeable channels that are primarily responsible for Ca2+ influx in non-excitable cells (1). Based on sequence similarity, this family can be further divided into several subfamilies, including TRPC (canonical), TRPV (vanilloid), TRPM (melastatin), and TRPP (polycystin) subfamilies (2). All TRP channels are predicted to share the same topology: six transmembrane (TM) segments, a pore-loop situated between TM5 and TM6, and intracellular N- and C-terminal tails. Depending on the subfamily, the cytosolic tails contain common conserved motifs such as ankyrin, PDZ, and coiled-coil domains (2). Similarly to voltage-gated K+ channels, functional TRP channels are formed by homo- or heteromeric assembly of four subunits (3). Despite noticeable sequence similarity, TRP channels differ considerably in their selectivity and activation mechanisms even within subfamilies (1). TRPV4, a member of the vanilloid group that preferentially forms homotetramers (4), was initially identified as a channel activated by hypotonic cell swelling (5–7). Subsequently, it was shown to respond to other stimuli as well, including phorbol esters (8), arachidonic acid (9), heat (10), and mechanosensation (11, 12). Studies using knockout mice have revealed that TRPV4 is involved in the regulation of systemic toxicity and mechanosensation (13–15).

Most TRP channels are believed to function at the plasma membrane to mediate Ca2+ entry. Similarly to other membrane proteins, they are co-translationally translocated into the endoplasmic reticulum (ER), in the first stage of their secretory transport. Proteins transiting the ER undergo diverse modifications, such as glycosylation and disulfide bond formation. Importantly, the ER is also the place of protein folding and assembly of subunits into multimeric complexes (16). These processes are aided by numerous ER chaperones. Properly folded cargo proteins leave this compartment in COPII-coated vesicles. In contrast, non-native proteins are recognized by quality-control proteins and retained in the ER (16). Eventually, terminally misfolded cargo is degraded by the ER-associated degradation (ERAD) pathway. In this process, the cargo is retrotranslocated from the ER, polyubiquitinated, and degraded in the proteasome (17). Proteins with lesions in the luminal domains are recognized and processed by the ERAD-L (luminal) machinery, whereas membrane proteins with defective cytosolic domains become substrates of the ERAD-C (cytosolic) pathway (18). At present, the mechanisms governing selection of ERAD-L substrates are understood to some extent, but the recognition of cytosolic lesions remains rather obscure. One possible mechanism could involve retention of faulty proteins by exposure of short amino acid motifs, such as di-arginine motifs, that are normally hidden in the mature structure (19). In general, the quality control in the ER is a very precise process that, for certain proteins, is responsible for the degradation of the majority of synthesized polypeptides due to folding and assembly defects (16, 20).

Recent studies have shown that the cytosolic tails of TRP channels, and in particular the ankyrin repeats and coiled-coil regions, play an important role in their folding, assembly, and degradation. In particular, TRPV4, which is involved in the regulation of systemic toxicity and mechanosensation (13–15), is a member of the vanilloid subfamily that preferentially forms homotetramers (4). It has been shown that TRPV4 is involved in the regulation of systemic toxicity and mechanosensation (13–15).
domains, are important for subunit interactions and the assembly of mature tetramers (21–27). For example, TRPV4 splice variants lacking parts of ankyrin repeats are retained in the ER as a result of their inability to oligomerize (21). However, much less is known about the auxiliary proteins regulating TRP channel maturation and their transit through the ER. Here we report a novel interaction between TRPV4 and human OS-9, a ubiquituous protein originally identified as being amplified in certain osteosarcomas (28). Interestingly, its closest relative in the yeast *Saccharomyces cerevisiae*, termed Yos9p, localizes to the ER lumen and plays an important role in the selection of substrates for the ERAD-L pathway (29, 30). In contrast, mammalian OS-9 localizes to the cytoplasmic side of the ER (31). In this work, we have found that human OS-9 binds the juxtamembrane region in the N-terminal tail of TRPV4, preferentially associating with TRPV4 monomers rather than tetramers. OS-9 prevents the polyubiquitination of TRPV4 at the ER and retards its transport through this compartment. Our data point to a role of OS-9 in the regulation of TRPV4 biogenesis by holding and protecting its monomers from a premature polyubiquitination and proteasomal degradation.

**EXPERIMENTAL PROCEDURES**

**Yeast Two-hybrid Screen**—The screen was performed using the Matchmaker 3 system (Clontech) according to the manufacturer’s protocol. The yeast strain AH109 expressing the N-terminal tail of mouse TRPV4 fused to the GAL4 DNA-binding domain (BD) was transformed with the human adult kidney cDNA library (Clontech) constructed in the pACT2 vector. Approximately 5 × 10⁶ transformants were screened on plates lacking histidine, adenine, and supplemented with 8 mM 3-aminotriazole. The cDNA clones isolated from growing colonies were subsequently reintroduced into the same yeast strain, and the strength of interactions was tested on plates containing asparagine-97 (Molecular Probes, Madison, WI), -ubiquitin P4D1 (Covance), rabbit -OS-9 antibody was kindly provided by L. Litovchick (31). The following commercially available antibodies were used: -FLAG M2 and -actin (Sigma-Aldrich), -V5 (Serotec), -GFP (Santa Cruz Biotechnology, Santa Cruz, CA), -ubiquitin P4D1 (Covance), rabbit -OS-9 (Protein Tech Group), -transferrin receptor (Zymed Laboratories), -Golin-97 (Molecular Probes, Madison, WI), -calnexin (Stressgen), and -HA 12CA5 (Roche Applied Science). Secondary horseradish peroxidase-coupled antibodies against guinea pig, rabbit, and mouse IgG were from Dako and GE Healthcare.

**Plasmids**—All plasmids containing mouse TRPV4 sequences were generated from the pcDNA3-TRPV4 plasmid provided by U. Wissenbach (7). The following plasmids used in this work were described previously: FLAG-N-TRPV4 (also named F9-N-TRPV4), TRPV4-FLAG, TRPV4–V5/His, TRPC4–V5/His, TRPV1–V5/His, TRPP2–V5/His, and TRPV4–V5 loop (32). TRPV4–V5/His Δ226–437, Δ40–235, and Δ40–112 are derivatives of TRPV4–V5/His and lack the indicated regions. Human α-subunit of ENaC was C-terminally tagged with V5/His in the pcDNA6 vector (Invitrogen). FLAG-tagged N-terminal fragments of TRPV4 (D1–D5) are derivatives of FLAG-N-TRPV4. The fusion of GAL4 DNA-BD with TRPV4 (aa 61–467) was created in the pGBK1T7 vector (Clontech). Fragments C1 (aa 307–667, Δ456–470) and C3 (aa 430–667) of human OS-9, isolated in the two-hybrid screen, were recloned into pcDNA3 vector (Invitrogen) with N-terminal FLAG tag. Isoform 1 of mouse OS-9 with a C-terminal V5 tag was constructed in the pcDNA3 vector. For generation of cell lines stably expressing TRPV4, a retroviral plasmid was prepared in which TRPV4–V5 loop sequence was cloned into the pLXSN vector (Clontech). For knockdown experiments, the OS-9 targeting sequence AACATCATCCAGGACAGAG or the control sequence ACCGATGATGTCGTTTT was cloned into the pLVTH vector (Addgene plasmid 12262) (33). These two plasmids were used to create additional knockdown constructs, in which the original GFP coding sequence was exchanged for TRPV4–V5/His Δ40–235. Plasmids coding for isoform 1 and 2 of mouse OS-9 were kindly provided by L. Litovchick (31) and HA-tagged ubiquitin by D. Bohmann (34).

**Co-immunoprecipitation Assay**—Thirty-six hours after transfection, HEK 293T cells were lysed in the immunoprecipitation buffer (1% Triton X-100, 1% sodium deoxycholate, 150 mM NaCl, 50 mM Tris, pH 8.0) supplemented with protease inhibitor mixture (Roche Applied Science). The lysates were cleared by centrifugation at 15,000 × g for 15 min at 4 °C. The supernatants were incubated for 1 h at 4 °C with an appropriate antibody, and subsequently with 30 μl of Protein G-Sepharose beads for ~3 h. The beads were washed extensively with the immunoprecipitation buffer, and the retained proteins were analyzed by Western blotting.

**Cross-linking**—Cells were lysed in the buffer containing 90 mM NaCl, 50 mM NaF, 5 mM Na₃P₂O₇, 1% Triton X-100, 20 mM Heps, pH 7.5, supplemented with protease inhibitor mixture (Roche Applied Science), and the lysates were cleared by centrifugation. The proteins were cross-linked with amine-reactive BS3 (Pierce) at a final concentration of 75–200 μM for 30 min at room temperature. The reaction was quenched with 40 mM Tris (pH 7.5) for 15 min.

**Cell Cultures, Transfections, and Viral Gene Transfers**—Human embryonic kidney (HEK 293T) and HeLa cells were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum. The human sarcoma cells SJSA-1 were cultured in RPMI 1640 with 10% fetal bovine serum. Transient transfections were carried out using the calcium phosphate method or FuGENE 6 reagent (Roche Applied Science). Stable polyclonal cell lines were created by transduction of SJSA-1 cells with retroviruses or lentiviruses, produced in HEK 293T cells, in the presence of 8 μg/ml Polybrene (Sigma-Aldrich). Cells stably expressing TRPV4–V5 loop protein were selected with 0.25 mg/ml Geneticin (Invitrogen).

**Reagents**—MG132 (Calbiochem) was used at 5 μg/ml. The rabbit α-TRPV4 polyclonal antibody was described previously (32). The guinea pig α-OS-9 antibody was kindly provided by L. Litovchick (31). The following commercially available antibodies were used: α-FLAG M2 and α-actin (Sigma-Aldrich), α-V5 (Serotec), α-GFP (Santa Cruz Biotechnology, Santa Cruz, CA), α-ubiquitin P4D1 (Covance), rabbit α-OS-9 (Protein Tech Group), α-transferrin receptor (Zymed Laboratories), α-Golin-97 (Molecular Probes, Madison, WI), α-calnexin (Stressgen), and α-HA 12CA5 (Roche Applied Science). Secondary horseradish peroxidase-coupled antibodies against guinea pig, rabbit, and mouse IgG were from Dako and GE Healthcare.
OS-9 Regulates TRPV4 Biogenesis

**Ubiquitination Assay**—Transfected HEK 293T or SJA-1 cells were washed with PBS and lysed in buffer A (8 M urea, 100 mM NaH_{2}PO_{4}, 1% Triton X-100, 10 mM Tris, pH 8.0; all steps were performed at room temperature). The supernatant obtained after centrifugation of the lysates at 75,000 × g was incubated with Ni^{2+}-nitrilotriacetic acid agarose (Qiagen) for 1 h. The beads were washed twice with buffer A and twice with buffer B (same as A, except for 0.5% Triton X-100 and pH 6.3). Bound proteins were eluted with buffer C (same as A, except for 0.1% Triton X-100 and pH 4.5).

**Immunofluorescence**—Cells were fixed in 3.7% paraformaldehyde in PBS for 10 min, permeabilized with 0.05% Triton X-100, and blocked in PBS containing 1% horse serum. Immunostainings were performed sequentially with appropriate primary antibodies and fluorescein labeled secondary antibodies. All images were obtained with an LSM 510 confocal microscope (Zeiss, Germany).

**Enzyme-linked Immunosorbent Assay**—The assay was performed essentially as described in a previous study (32). The cells split in parallel were lysed with a lysis solution (Tropix, Bedford, MA) to measure the β-galactosidase activity with o-nitrophenyl-β-d-galactopyranoside as the substrate. This reaction was performed to control the transfection efficiency and normalize the alkaline phosphatase activity.

**Biotinylation Assay**—Transfected HEK 293T cells were washed three times with ice-cold PBS (pH 8.0) and collected by centrifugation at 1500 rpm for 5 min. Surface proteins were biotinylated with 1 mg/ml sulfo-NHS-LC-biotin (Pierce) in PBS (pH 8.0) at room temperature for 30 min, according to the manufacturer’s recommendations. Subsequently, the cells were lysed at 4 °C for 1 h in PBS (pH 8.0) containing 1% Triton X-100, 5 mM EDTA, and protease inhibitors (Roche Applied Science). Cell lysates were incubated with 30 μl of streptavidin beads (Pierce) at 4°C overnight. The beads were washed twice with 1 M KCl, twice with 0.1 M Na_{2}CO_{3}, and once with PBS. Biotinylated proteins were eluted with SDS-PAGE loading buffer and analyzed by immunoblotting with anti-V5 and anti-transferrin receptor antibodies.

**Zebrafish Experiments**—Wild-type zebrafish ABTL strain was maintained as described before (35). Embryos were kept in Danieau solution and staged according to hours post-fertilization. The capped RNAs, synthesized using the mMessage mMACHINE T7 kit (Ambion, Austin, TX), were microinjected into one-cell stage embryos in 100 mM KCl, 0.1% Phenol Red, and 10 mM HEPES at doses of 600 (TRPV4), 1000 (TRPC4), or 200 (OS-9) pg. Data were analyzed with the Cochran-Mantel-Haenszel test.

RESULTS

**OS-9 Is a Novel TRPV4-binding Protein**—To identify new proteins interacting with the intracellular N-terminal tail of TRPV4 (N-TRPV4), we performed a yeast two-hybrid assay. Screening of a human adult kidney cDNA library resulted in the isolation of 16 clones that specifically provided adenine and histidine prototrophy to the yeast when co-expressed with the GAL4 DNA-binding domain fused to aa 61–467 of mouse...
TRPV4. Three of these clones contained overlapping fragments originating from the same cDNA sequence. The protein encoded by this cDNA was originally identified as amplified in osteosarcomas and termed OS-9 (28). Three splicing isoforms of human OS-9 were initially described (36). The longest isoform, 1, contains 667 aa, isoform 2 lacks aa 535–589, whereas isoform 3 lacks aa 456–470 and 535–589. All three isoforms isolated by us code for the C-terminal domain of OS-9, starting at different residues. Clone OS-9-C1 extended from aa 307, OS-9-C2 from aa 395, and OS-9-C3 from aa 430. The sequence analysis revealed that OS-9-C2 and OS-9-C3 represent isoform 1 of human OS-9, whereas clone OS-9-C1, lacking aa 456–470 but not aa 535–589, conforms to the recently discovered fourth isoform (GenBank™ accession number NP_001017958).

To confirm that the interaction between OS-9 and TRPV4 also occurs in mammalian cells, OS-9-C1 and OS-9-C3 fragments were N-terminally fused to the FLAG tag, expressed in HEK 293T cells, and purified using anti-FLAG antibodies. The full-length TRPV4 co-immunoprecipitated with both proteins, but not with FLAG-tagged GFP (Fig. 1A). In a reverse approach, we found that the isoforms 1 and 2 of mouse OS-9 co-immunoprecipitated with FLAG-tagged full-length TRPV4 (TRPV4-FLAG) as well as with FLAG-tagged N-terminal tail of TRPV4 (FLAG-N-TRPV4 (Fig. 1B)). We subsequently decided to map the region of TRPV4 responsible for the interaction with OS-9. For this purpose, we generated additional FLAG-tagged N-terminal fragments of TRPV4 and found that a fragment covering all three ankyrin domains and the juxtamembrane region (fragment D5, aa 237–468) interacted most strongly with OS-9. Fragments containing aa 1–370 (D2) and aa 1–436 (D3) did not show any interaction (Fig. 1C). However, the co-immunoprecipitation of OS-9 was regained when the D2 fragment was extended by aa 438–468 (D4). This experiment indicates that the juxtamembrane region (aa 438–468) in TRPV4 is required, although not necessarily sufficient, for the interaction with OS-9.

OS-9 was previously isolated in three two-hybrid screens using unrelated proteins as baits (31, 37, 38). Interestingly, we have found that all these proteins share a short stretch of limited sequence similarity in their predicted OS-9 interacting regions (Fig. 1D). For TRPV4, this region of similarity extends from aa 441 to 466 and may be part of the OS-9 binding interface.

**OS-9 Interacts with Other TRP Family Members**—To determine whether OS-9 interacts with other channel proteins, we performed a co-immunoprecipitation assay in HEK 293T cells, using several V5/His-tagged TRP proteins (TRPV1, TRPV4, TRPC4, and TRPP2), as well as the identically tagged α-subunit of the epithelial sodium channel (αENaC). This experiment showed that OS-9 co-immunoprecipitated only with TRPV4 and TRPV1, but not with other tested TRP channels or αENaC (Fig. 2). Importantly, TRPV1 and TRPV4 show high sequence conservation (62% identity) within the proposed OS-9 binding region (Fig. 1D). Thus, OS-9 may specifically bind the TRP channels of the vanilloid subfamily.

**OS-9 Interacts with TRPV4 at the ER**—The splice variants of TRPV4 that contain deletions within the ankyrin repeats are retained in the ER (21). In agreement with this report, we observed that a TRPV4 mutant missing all three repeats (D226–437) localized to the ER (Fig. 3A). The same localization was found for another TRPV4 mutant, in which a more proximal region was deleted (D40–235, Fig. 3A). In contrast, a TRPV4 variant with a shorter truncation (Δ40–112) did not accumulate in the ER but instead was detected at the cell periphery (Fig. 3A). Interestingly, both ER-retained mutants immunoprecipitated OS-9 more efficiently than the full-length TRPV4 did, suggesting that the interaction between the two proteins takes place at the ER (Fig. 3A). This hypothesis was supported by a previously reported finding that OS-9 associates with the cytoplasmic side of the ER (31). Thus, we decided to investigate whether OS-9 plays a role in the secretory transport of TRPV4 and affects its cellular distribution. Transiently expressed OS-9 extensively co-localized with the ER marker BAP31 in HeLa cells (Fig. 3B). Similar results were obtained for endogenous OS-9 in SJSA-1 cells (Fig. 3C), an osteosarcoma cell line that expresses high levels of OS-9 (36). Using a retroviral gene transfer, we prepared a polyclonal SJSA-1 cell line stably expressing full-length TRPV4 protein with a V5 tag engineered into its first extracellular loop (TRPV4-V5 loop) (32). In these cells, a substantial portion of TRPV4 accumulated in the ER, as demonstrated by its co-localization with OS-9 and BAP31 (Fig. 3D).

**Depletion of OS-9 Facilitates Release of TRPV4 from the ER**—Next we investigated whether depletion of endogenous OS-9 by RNA interference affects the subcellular localization of TRPV4–V5 loop protein in SJSA-1 cells. We identified one shRNA clone that efficiently reduced OS-9 protein levels in a transient expression system (data not shown) and generated a lentivirus that directed the expression of this shRNA in combination with GFP. Transduction of SJSA-1 cells with this lentivirus resulted in a substantial reduction of OS-9 protein, as assessed by Western blot analysis (Fig. 4A), and attenuated the

---

36564 JOURNAL OF BIOLOGICAL CHEMISTRY

VOLUME 282 • NUMBER 50 • DECEMBER 14, 2007

In Ref. 36, the SJSA-1 cell line is called OsA-CL.
OS-9 Regulates TRPV4 Biogenesis

**FIGURE 3. OS-9 interacts with TRPV4 at the endoplasmic reticulum.** A, OS-9 interacts more strongly with the ER-retained TRPV4 truncations than with the full-length TRPV4. HEK 293T cells were transfected with plasmids encoding V5/His-tagged TRPV4 variants: wild-type (WT), Δ40–235, Δ226–437, or V5-tagged GFP, as well as the isoform 1 of OS-9. The proteins purified with anti-V5 antibodies were analyzed by Western blotting. The immunostainings show the prominent ER localization of the TRPV4–V5/His Δ226–437 (upper row) and Δ40–235 (middle row) variants in comparison to the peripheral staining of the Δ40–112 truncation (lower row). TRPV4 proteins were immunodecorated with anti-V5 antibodies and anti-mouse Alexa488-coupled antibodies (green), and the ER was visualized with anti-calnexin antibodies and anti-rabbit Cy3-coupled antibodies (red). B, OS-9 co-localizes with the ER marker BAP31 in HeLa cells. The cells were transfected with plasmids encoding V5-tagged isoform 1 of OS-9 (OS-9 iso1) and BAP31-GFP, an ER marker (green). OS-9 was visualized by immunofluorescence using anti-V5 antibodies and anti-mouse Cy3-coupled antibodies (red). Merged images are shown in the rightmost panel. C, endogenous OS-9 in SJSA-1 cells, immunodecorated with the rabbit anti-OS-9 antibodies (red), shows apparent ER localization. The ER was visualized with BAP31-GFP (green). Merged images are shown in the rightmost panel. D, OS-9 and TRPV4 co-localize with BAP31 in the ER of SJSA-1 cells. SJSA-1 cells stably expressing the TRPV4–V5 loop protein were transiently transfected with a plasmid encoding BAP31-GFP (blue). OS-9 was immunodecorated with rabbit anti-OS-9 antibodies followed by anti-rabbit Cy3-coupled antibodies (green). TRPV4 was stained with anti-V5 antibodies followed by anti-mouse Cy5-coupled antibodies (red). A merge of the stainings is shown in the rightmost panel. The insets show enlarged portions of the main images.

OS-9 immunoreactivity in GFP-positive (i.e. transduced) cells (Fig. 4B, upper panels). Transduction with the lentivirus expressing control shRNA had no effect on OS-9 levels (Fig. 4, A and B). We next analyzed the immunolocalization of TRPV4 in GFP-positive SJSA-1 cells (Fig. 4C). We found that TRPV4 did not substantially co-localize with the ER resident protein calnexin in cells expressing the OS-9 shRNA, but instead partially overlapped with Golgin-97, a marker of Golgi stacks. In contrast, TRPV4 continued to accumulate in the calnexin-positive compartment in cells expressing the control shRNA. These findings suggest that OS-9 controls the transit of TRPV4 from the ER to the Golgi.

**OS-9 Diminishes the Abundance of TRPV4 at the Plasma Membrane**—The control of TRPV4 exerted by OS-9 at the ER could affect the presence of TRPV4 at the plasma membrane, a functional site for this channel. To investigate this possibility, we transiently transfected HEK 293T cells with a plasmid encoding TRPV4–V5 loop protein. The abundance of this protein at the cell surface was quantified using an enzyme-linked immunosorbent assay, following the incubation of non-permeabilized cells with anti-V5 antibodies (32). Because endogenous OS-9 cannot be detected with specific antibodies in the lysates of HEK 293T cells (Ref. 31 and data not shown), we used a co-expression approach. As shown in Fig. 5A, mouse OS-9 led to a significant decrease in the TRPV4 immunoreactivity at the plasma membrane, whereas its total amount was not affected by OS-9. The decrease in TRPV4 surface expression in the presence of OS-9 was also confirmed by cell surface biotinylation (Fig. 5B). Whereas TRPV4 was detected in the fraction of biotinylated proteins only in the absence of co-expressed OS-9, endogenous transferrin receptor was biotinylated regardless of OS-9 expression. Together, these experiments demonstrate that OS-9 diminishes the abundance of TRPV4 ion channel at the plasma membrane.

**OS-9 Rescues the Adverse Effects of TRPV4 Expression during Zebrafish Development**—To investigate the regulation of TRPV4 by OS-9 in vivo, we used zebrafish as a model organism. Ectopic expression of mouse TRPV4 in zebrafish causes severe developmental abnormalities (Fig. 6A). The abnormal phenotypes caused by the injection of TRPV4 mRNA into zebrafish embryos (n = 226) could be classified into three dysmorphic classes: class 1 (27%) with a curved or shortened body axis and pericardial or periocular edema, class 2 (11%) with an extremely shortened body axis and eye malformation (small eye or only one eye), and class 3 (12%) scarcely having a body axis, eyes, or brain. In addition, a substantial fraction of injected embryos (17%) died within 48 h post fertilization. Because we noted that TRPV4 expression decreases survival of HEK 293T cells,5 we hypothesized that these abnormalities are caused by an uncontrolled accumulation and activation of TRPV4 at the cell surface, compromising normal calcium homeostasis. We postulated that OS-9 should reverse the developmental abnormalities associated with the ectopic TRPV4 expression. Zebrafish embryos were therefore injected with either TRPV4 mRNA alone, or in combination with OS-9 mRNA. As shown in Fig. 6B, coinjection of OS-9 mRNA significantly ameliorated the TRPV4-associated abnormalities (dysmorphic class 1: 27%, class 2: 11%, and class 3: 12%).

---

5 Y. Wang and T. Wegierski, unpublished observation.
OS-9 Regulates TRPV4 Biogenesis

FIGURE 4. Knockdown of OS-9 in SJSA-1 cells releases TRPV4 from the ER. A, transduction of SJSA-1 cells with a lentivirus directing the expression of the OS-9-specific shRNA, as well as GFP, leads to a substantial decrease in the endogenous OS-9 levels. Cell lysates from transduced and wild-type SJSA-1 cells were analyzed by Western blotting using rabbit anti-OS-9 and mouse anti-GFP antibodies. The amounts of both isoform 1 and 2 of OS-9 were reduced in cells expressing the OS-9 shRNA, as compared with wild-type (WT) cells or cells expressing control shRNA. The equal loading of proteins was controlled with anti-actin antibodies. B, transduced SJSA-1 cells were visually identified on the basis of GFP fluorescence (green), and the OS-9 stainings were examined following immunodecoration with rabbit anti-OS-9 antibodies and anti-rabbit Cy3-coupled antibodies (red). Cells expressing the OS-9 shRNA (a) showed markedly reduced OS-9 signals in comparison to cells expressing control shRNA (b) or to non-transduced (GFP-negative) cells. C, OS-9 knockdown releases TRPV4 from the ER. The SJSA-1 cells stably expressing the TRPV4–V5 loop protein, were transduced with lentiviruses directed expression of the OS-9–specific (two upper rows of immunostainings, a and b) or control (bottom row, c) shRNAs (identified as GFP-positive cells). The cells were analyzed by immunofluorescence using either mouse anti-V5 antibodies and anti-mouse Cy3-coupled antibodies (red) in combination with rabbit anti-calnexin (ER marker) antibodies and anti-rabbit Cy5-coupled antibodies (green, a and c), or using rabbit anti-TRPV4 antibodies and anti-rabbit Cy3-coupled antibodies (red) in combination with mouse anti-Golgin-97 (Golgi stacks marker) antibodies and anti-mouse Cy5-coupled antibodies (green, b). Merge of images (not including GFP signals) is shown in the rightmost panels. TRPV4 substantially co-localized with calnexin in cells expressing the control shRNA but not in cells expressing the OS-9 shRNA. Instead, in the latter cells, TRPV4 displayed partial co-localization with Golgin-97.

23%; class 2: 6%; class 3: 7%; and lethal: 7%). In contrast, co-injection of OS-9 mRNA was not effective at preventing similar developmental defects caused by the ectopic expression of TRPC4 (Fig. 6B), a TRP channel that does not detectably interact with OS-9 in HEK 293T cells (Fig. 2). These findings demonstrate that OS-9 can control TRPV4 function in a complex model organism.

OS-9 Prevents TRPV4 Polyubiquitination at the ER—The protein most closely related to the mammalian OS-9 in yeast, Yos9p, functions in the ERAD of misfolded proteins (29). Therefore, we investigated whether OS-9 performs a similar function in mammalian cells. Cargo proteins, which fail to fold and/or assemble properly in the ER, are marked with polyubiquitin chains for degradation in the proteasome (17). We found that transiently expressed TRPV4 in HEK 293T cells can be modified by ubiquitin attachment. The basal level of ubiquitination is increased following the treatment with the proteasome inhibitor MG132 (Fig. 7A). Importantly, this modification is substantially more pronounced for TRPV4 truncations that are retained in the ER: Δ40–235 (Fig. 7B) and Δ226–437 (data not shown). These data suggest that misfolded and/or unassembled TRPV4 subunits are subject to polyubiquitination at the ER and subsequent degradation in the proteasome. Surprisingly, co-expression of OS-9 led to a dramatic reduction in the ubiquitination level of TRPV4, both in the absence and the presence of proteasome inhibitors (Fig. 7, A and B). In contrast, OS-9 did not affect the ubiquitination of the transiently expressed αENaC (Fig. 7C), which also accumulated in the ER (data not shown). αENaC, when expressed alone, is polyubiquitinated and degraded in the proteasome as a result of its inability to assemble with the missing subunits of the channel (39). Thus, OS-9 appears to specifically protect TRPV4 from polyubiquitination and, presumably, from degradation in the ERAD pathway.

Subsequently, we investigated the ubiquitination status of stably expressed TRPV4 in SJSA-1 cells following knockdown of endogenous OS-9 by RNA interference. The ER-retained variant of TRPV4, Δ40–235, was chosen for this experiment, to uncouple the effects of OS-9 depletion on TRPV4 ubiquitination from TRPV4 release from the ER. The TRPV4–V5/His Δ40–235 protein, expressed together with either OS-9-specific or control shRNA, showed a predominant ER localization in SJSA-1 cells, irrespective of the OS-9 expression levels (supplemental Fig. S1). Consistent with the overexpression data, we found that the depletion of OS-9 led to a clear increase in the levels of ubiquitinated TRPV4, as compared with control cells, both in the presence and absence of proteasomal inhibitors (Fig. 7D).

OS-9 Associates Predominantly with the Monomeric Form of TRPV4—These results point to a role of OS-9 in protecting TRPV4 against polyubiquitination at the ER. We further hypothesized that the binding of OS-9 and its protective effect may be confined to the newly produced TRPV4 proteins that have not completed the oligomerization process. Therefore, we investigated the binding preference of OS-9 protein toward monomers and tetramers of TRPV4. We used the chemical cross-linker, BS3, to stabilize the oligomeric forms of TRPV4. As shown in Fig. 8A, addition of BS3 led to the appearance of additional high molecular weight species of TRPV4 in a dose-dependent manner. These forms are likely to represent covalently bound dimers, trimers, and tetramers of TRPV4 channel. In addition, the cross-linked subunits of V5/His-tagged TRPV4 co-immunoprecipitated with TRPV4-FLAG.
and not with FLAG-tagged GFP (Fig. 8A). These results suggest that the co-expressed TRPV4–V5/His and TRPV4–V5 loop subunits jointly form the TRPV4 tetrameric channels. As a next step, we co-expressed TRPV4–V5/His either with FLAG-tagged OS-9-C1 (FLAG-OS-9) or with control FLAG-tagged proteins in HEK 293T cells. The proteins in the cell lysates were cross-linked with BS3 and purified with anti-FLAG antibodies. As expected, TRPV4–FLAG efficiently immunoprecipitated cross-linked oligomers of TRPV4–V5/His (Fig. 8B). TRPV4–V5/His was also co-immunoprecipitated with FLAG-OS-9 and FLAG-tagged N-terminal tail of TRPV4 (FLAG-N-TRPV4), but not with FLAG-GFP. However, whereas both monomeric and oligomeric forms of TRPV4–V5/His co-purified with FLAG-N-TRPV4 in comparable amounts, only the monomeric form was detectable in the purified fraction of FLAG-OS-9 (Fig. 8B). These results indicate that the N-terminal tail of TRPV4 can associate with both monomers and tetramers of full-length TRPV4, but OS-9 predominantly interacts with TRPV4 monomers. In a different approach, FLAG-OS-9 and TRPV4–V5/His proteins were expressed independently and only the TRPV4–V5/His-containing cell lysate was subjected to cross-linking. Under such conditions, purified FLAG-OS-9 pulled down only TRPV4 monomers, indicating that TRPV4 tetramers are not recognized by OS-9 (Fig. 8C).

DISCUSSION

In this work we report that OS-9 is a novel TRPV4-interacting protein. The interaction takes place at the ER, involving the C-terminal domain of OS-9 and the juxtamembrane region in the N-terminal tail of the TRPV4 monomer. We have found that depletion of OS-9 by RNA interference facilitates the release of TRPV4 from the ER and advances TRPV4 through the secretory pathway. Consistently, co-expression of both proteins leads to a decreased localization of the channel at the plasma membrane in cultured cells and reduces TRPV4-mediated toxicity in a complex model organism. Although we expected OS-9 to target TRPV4 for the ERAD pathway, we found that OS-9 efficiently blocks TRPV4 polyubiquitination at the ER.

The assembly and folding of channels is accomplished in the ER following co-translational translocation of their individual subunits (40). The best understood example of a hexahelical
channel formation is represented by the Shaker voltage-dependent K⁺ channel. The oligomerization of this channel is driven by the N-terminal tetramerization domain, and distinct assembly and folding steps are interspersed in the ER (41, 42). TRP proteins, which belong to the same hexahedral cation channel superfamily, are believed to assemble into functional homo- or heterotetramers, but the mechanisms governing their assembly are currently not well understood (3). Recent studies have implicated diverse parts of TRP proteins in this process, including both N- and C-terminal cytosolic tails, as well as the transmembrane segments (4, 43). However, it now appears that two motifs, conserved within distinct subfamilies, may be primarily responsible for TRP subunit oligomerization and its specificity: coiled-coil domains and ankyrin repeats (21–27). In addition to causing defects in subunit joining, deletions or mutations within ankyrin repeats or coiled-coil domains frequently lead to the ER retention and/or decreased cell surface localization of TRP proteins (21, 23, 24, 26, 27). For example, three splice variants of TRPV4 lacking parts of ankyrin repeats are unable to oligomerize and, as a result, are retained in the ER (21). In support of this finding, we have found that a TRPV4 truncation lacking all three ankyrin repeats Δ226–437, as well as another variant containing a deletion in the upstream region Δ40–235, also accumulates in the ER. The entrapment of the two TRPV4 truncations in the ER most likely results from their inability to assemble and/or correctly fold, an explanation supported by their polyubiquitination status. Although OS-9 can delay the passage of the full-length TRPV4 through the ER, the following observations argue against a role of OS-9 in the ER retention of the two truncated variants. First, TRPV4 Δ40–235 localizes in the ER of SJSA-1 cells following knockdown of OS-9. Second, OS-9 has a strong inhibitory effect on the polyubiquitination of TRPV4 at the ER and is therefore unlikely to function as a bona fide quality-control protein (discussed below).

Recently we have reported that TRPV4 is multiubiquitinated and internalized from the plasma membrane in the presence of AIP4 ubiquitin ligase (32). The AIP4-mediated multiubiquitination was distinct from the polyubiquitination of TRPV4, which we also observed. Here, we show that the polyubiquitination is more pronounced for the ER-retained variants of TRPV4 and that the inhibition of proteasomal activity leads to the increased accumulation of polyubiquitinated TRPV4. Thus, this polyubiquitination likely represents the modification of TRPV4 subunits that are targeted for the ERAD pathway. Importantly, we have found that OS-9 strongly diminishes the polyubiquitination of both wild-type TRPV4 and its ER-retained variants. In contrast, OS-9 neither interacts with nor affects ubiquitination of the ER-retained α subunit of the epithelial sodium channel. Therefore, the effect of OS-9 does not result from inhibition of the ERAD ubiquitination machinery but requires binding to TRPV4 and, perhaps, involves the competition with an ubiquitin ligase for the same site in the channel. Furthermore, the ubiquitination of the ER-retained variant of TRPV4 was increased in SJSA-1 cells depleted of OS-9, corroborating the previous findings.
Our immunoprecipitation and pulldown experiments with the cross-linked TRPV4 demonstrate that OS-9 efficiently interacts with TRPV4 monomers, but not with tetramers. These results suggest that OS-9 associates with TRPV4 at an early stage of TRPV4 maturation in the ER and may dissociate from it just prior or during the oligomerization. These data are consistent with a relatively strong binding of OS-9 to the ER-retained, immature variants of TRPV4. Thus, OS-9 may potentially function as a chaperone-like protein that associates with TRPV4 monomers or its translocating nascent chains. OS-9 could protect the N-terminal tail of TRPV4 against a premature polyubiquitination until the C-terminal tail becomes ready for oligomerization of the channel. In fact, the cooperation of both cytosolic termini in TRPV4 assembly has been recently proposed (4). Furthermore, the C-terminal tail comprises an assembly domain in TRPV1 (26), the closest relative of TRPV4, which also interacts with OS-9 (Fig. 2). Such a protective binding of OS-9 might be important to reduce the number of newly produced TRPV4 subunits targeted for ERAD. It has been estimated that 60–80% of immature subunits of wild-type cystic fibrosis transmembrane conductance regulator channel are quickly degraded with a half-life of 20–40 min (20). Moreover, the ubiquitination of cystic fibrosis transmembrane conductance regulator subunits appears to start before their translation is complete (44, 45).

In agreement with the above presented model, the delayed secretory transport of TRPV4, in the presence of OS-9, may be explained by the excessive binding of OS-9 to TRPV4 monomers. Although the chaperone-like function of OS-9 in TRPV4 maturation seems most plausible, we cannot exclude the possibility that OS-9 impedes the release of TRPV4 from the ER to negatively regulate its amount and activity at the plasma membrane. As evidenced by our experiments with cultured cells and zebrafish embryos, co-expression of OS-9 efficiently reduces the plasma membrane localization and toxicity of TRPV4, respectively.

OS-9 was previously reported to transiently bind a C-terminal tail of meprin β, a subunit of the heterodimeric membrane protease meprin B (31). Because this region of meprin β is required for the ER-to-Golgi transport of the protein, the authors suggested that OS-9 might be involved in this process. However, this hypothesis was not verified experimentally. In light of our results, it is possible that OS-9 binds meprin β subunits not to mediate their export but rather to protect them against premature ubiquitination. In addition, OS-9 was found to interact...
OS-9 Regulates TRPV4 Biogenesis

with two proteins that lack any transmembrane domains: N-copine and the α subunit of HIF-1 transcription factor (37, 38). Semenza and colleagues have shown that OS-9 acts as an adaptor molecule between HIF-1α and prolylhydroxylases to promote hydroxylation of the transcription factor (37), suggesting that OS-9 engages in more than one cellular function. Further work is required to establish the common denominator of different OS-9 functions and its ability to bind both soluble and membrane protein targets.

Interestingly, a putative yeast homolog of OS-9, Yos9p, is a lectin-like protein involved in the selection of terminally misfolded glycoproteins for the ERAD-L pathway (29). However, the sequence similarity between the mammalian and yeast proteins is mostly confined to their N-terminal regions, encompassing the mannose 6-phosphate receptor homology domain that is essential for the lectin activity of Yos9p (29). The putative homologs also differ in their cellular localization (see the introduction). Finally, our results suggest that mammalian OS-9, in contrast to Yos9p, is not involved in the quality control of ER cargo proteins. Therefore, although it cannot be excluded that Yos9p and OS-9 share some of their functions, they unlikely represent true orthologs.

In summary, our work demonstrates that OS-9 binds TRPV4 at the ER to regulate its biogenesis and passage through this compartment, strengthening the recent hypothesis that OS-9 plays a role as an auxiliary ER protein for select classes of membrane cargo proteins (31).

Acknowledgments—We thank Drs. L. Litovchick, D. Bohmann, and D. Trono for reagents, R. Müller for excellent technical assistance, and members of the Walz laboratory for helpful discussions.

REFERENCES

1. Montell, C., Birnbaumer, L., and Flockerzi, V. (2002) Cell 108, 595–598
2. Clapham, D. E. (2003) Nature 426, 517–524
3. Schaefer, M. (2005) Pflugers Arch. 451, 35–42
4. Hellwig, N., Albrecht, N., Harteneck, C., Schultz, G., and Schaefer, M. (2005) J. Cell Sci. 118, 917–928
5. Liedtke, W., Choe, Y., Marti-Renom, M. A., Bell, A. M., Denis, C. S., Sali, A., Hudspeth, A. J., Friedman, J. M., and Heller, S. (2000) Cell 103, 525–535
6. Strotmann, R., Harteneck, C., Unnennacher, K., Schultz, G., and Plant, T. D. (2000) Nat. Cell Biol. 2, 695–702
7. Wissenbach, U., Boding, M., Freichel, M., and Flockerzi, V. (2000) FEBS Lett. 485, 127–134
8. Watanabe, H., Davis, J. B., Smart, D., Jerman, J. C., Smith, G. D., Hayes, P., Vriens, J., Cairns, W., Wissenbach, U., Prener, J., Flockerzi, V., Droogmans, G., Benham, C. D., and Niemeyer, B. A. (2002) J. Biol. Chem. 277, 13569–13577
9. Watanabe, H., Vriens, J., Prener, J., Droogmans, G., Voets, T., and Niemeyer, B. (2003) Nature 424, 434–438
10. Guler, A. D., Lee, H., Ida, T., Shimizu, I., Tominaga, M., and Caterina, M. (2002) J. Neurosci. 22, 6408–6414
11. Gao, X., Wu, L., and O’Neil, R. G. (2003) J. Biol. Chem. 278, 27129–27137
12. Liedtke, W., Tobin, D. M., Bargmann, C. I., and Friedman, J. M. (2003) Proc. Natl. Acad. Sci. U. S. A. 100 Suppl 2, 14531–14536
13. Mizuno, A., Matsumoto, N., Imai, M., and Suzuki, M. (2003) Am. J. Physiol. 285, C96–C101
14. Suzuki, M., Mizuno, A., Kodaira, K., and Imai, M. (2003) J. Biol. Chem. 278, 22664–22668
15. Liedtke, W., and Friedman, J. M. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 13698–13703
16. Ellgaard, L., and Helenius, A. (2003) Nat. Rev. Mol. Cell Biol. 4, 181–191
17. Meusser, B., Hirsch, C., Jarosch, E., and Sommer, T. (2005) Nat. Cell Biol. 7, 766–772
18. Vashist, S., and Ng, D. T. (2004) J. Cell Biol. 165, 41–52
19. Zerangue, N., Schwappach, B., Jan, Y. N., and Jan, L. Y. (1999) Neuron 22, 537–548
20. Kopito, R. R. (1999) Physiol. Rev. 79, S176–S173
21. Arniges, M., Fernandez-Fernandez, J. M., Albrecht, N., Schaefer, M., and Valverde, M. A. (2006) J. Biol. Chem. 281, 1580–1586
22. Erler, J., Hinret, D., Wissenbach, U., Flockerzi, V., and Niemeyer, B. A. (2004) J. Biol. Chem. 279, 34456–34463
23. Erler, J., Al-Ansary, D. M., Wissenbach, U., Wagner, T. F., Flockerzi, V., and Niemeyer, B. A. (2006) J. Biol. Chem. 281, 38396–38404
24. Engelke, M., Friedrich, O., Budde, P., Schaefer, C., Niemann, U., Zitt, C., Jungling, E., Rocks, O., Luckhoff, A., and Frey, J. (2002) FEBS Lett. 523, 193–199
25. Garcia-Sanz, N., Fernandez-Carvajal, A., Morcilla-Palao, C., Planelles-Cases, R., Fajardo-Sanchez, E., Fernandez-Ballester, G., and Ferrer-Montiel, A. (2004) J. Neurosci. 24, 5307–5314
26. Mei, Z. Z., Xie, R., Beech, D. J., and Jiang, L. H. (2006) J. Biol. Chem. 281, 38748–38756
27. Su, Y. A., Hutter, C. M., Trent, J. M., and Meltzer, P. S. (1996) Mol. Carcinog. 15, 270–275
28. Coullier, J. H., Pearse, B. R., and Hebert, D. N. (2005) Mol. Cell 19, 717–719
29. Friedmann, E., Salzberg, Y., Weinberger, A., Shaltiel, S., and Gerst, J. E. (2002) J. Biol. Chem. 277, 35274–35281
30. Liedtke, W., Friedmann, E., and Shaltiel, S. (2002) J. Biol. Chem. 277, 34413–34423
31. Wiznerowicz, M., and Trono, D. (2003) J. Virol. 77, 8957–8961
32. Treier, M., Staszewski, L. M., and Bohmann, D. (1994) Cell 78, 787–798
33. Westerfield, M. (1995) The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish (Danio rerio), 4th Ed., pp. 1.1–1.35, University of Oregon Press, Eugene, OR
34. Kimura, Y., Nakazawa, M., and Yamada, M. (1998) J. Biochem. (Tokyo) 123, 876–882
35. Baek, J. H., Mahon, P. C., Oh, J., Kelly, B., Krishnamachary, B., Pearson, M., Chan, D. A., Giaccia, A. J., and Semenza, G. L. (2005) Mol. Cell 17, 503–512
36. Nakayama, T., Yagi, M., Kuwajima, G., Yoshie, O., and Sakata, T. (1999) FEBS Lett. 453, 77–80
37. Staub, O., Gautschi, I., Ishikawa, T., Breitshopf, K., Cianchonav, A., Schild, L., and Rotin, D. (1997) EMBO J. 16, 6325–6336
38. Green, W. N., and Millar, N. S. (1995) Trends Neurosci. 18, 280–287
39. Li, M., Jan, Y. N., and Jan, L. Y. (1992) Science 257, 1225–1230
40. Schulteis, C. T., Nagaya, N., and Papazian, D. M. (1998) J. Biol. Chem. 273, 26210–26217
41. Chang, Q., Gyfogianni, E., van de Graaf, S. F., Hoefs, S., Weidema, F. A., Bindels, R. J., and Hoenderop, J. G. (2004) J. Biol. Chem. 279, 54304–54311
42. Younger, J. M., Chen, L., Ren, H. Y., Rossner, M. F., Turnbull, E. L., Fan, C. Y., Patterson, C., and Cyr, D. M. (2006) Cell 126, 571–582
43. Sato, S., Ward, C. L., and Kopito, R. R. (1998) J. Biol. Chem. 273, 7189–7192