The epithelial Ca\(^{2+}\) channels TRPV5 and TRPV6 mediate the Ca\(^{2+}\) influx in 1,25-dihydroxyvitamin \(D_3\)-responsive epithelia and are therefore essential in the maintenance of the body Ca\(^{2+}\) balance. These Ca\(^{2+}\) channels assemble in (hetero)tetrameric channel complexes with different functional characteristics regarding Ca\(^{2+}\)-dependent inactivation, ion selectivity, and pharmacological block. Glutathione S-transferase pull-downs and co-immunoprecipitations demonstrated an essential role of the intracellular N- and C-tails in TRPV5 channel assembly by physical interactions between N-N tails, C-C tails, and N-C tails. Patch clamp analysis in human embryonic kidney (HEK293) cells and \(45\text{Ca}^{2+}\) uptake experiments in Xenopus laevis oocytes co-expressing TRPV5 wild-type and truncated proteins indicated that TRPV5\(\Delta N\) (deleted N-tail) and TRPV5\(\Delta C\) (deleted C-tail) decreased channel activity of wild-type TRPV5 in a dominant-negative manner, whereas TRPV5\(\Delta N\Delta C\) (deleted N-tail/C-tail) did not affect TRPV5 activity. Oocytes co-expressing wild-type TRPV5 and TRPV5\(\Delta N\) or TRPV5\(\Delta C\) showed virtually no wild-type TRPV5 expression on the plasma membrane, whereas co-expression of wild-type TRPV5 and TRPV5\(\Delta N\Delta C\) displayed normal channel surface expression. This indicates that TRPV5 trafficking toward the plasma membrane was disturbed by assembly with TRPV5\(\Delta N\) or TRPV5\(\Delta C\) but not with TRPV5\(\Delta N\Delta C\). TRPV5 channel assembly signals were refined between amino acid positions 64–77 and 596–601 in the N-tail and C-tail, respectively. Pull-down assays and co-immunoprecipitations demonstrated that N- or C-tail mutants lacking these critical assembly domains were unable to interact with tails of TRPV5. In conclusion, two domains in the N-tail (residues 64–77) and C-tail (residues 596–601) of TRPV5 are important for channel subunit assembly, subsequent trafficking of the TRPV5 channel complex to the plasma membrane, and channel activity.

TRPV5 and TRPV6 constitute the Ca\(^{2+}\) influx pathway in 1,25-dihydroxyvitamin \(D_3\)-responsive epithelia, including small intestine, kidney, and placenta, and play a vital role in the process of Ca\(^{2+}\) (re)absorption (1–4). Both channels belong to a distinct subfamily (TRPV) within the superfamily of transient receptor potential channels (TRP).\(^1\) The TRP family consists of a diverse group of non-voltage-gated cation channels, including TRPC (canonical), TRPM (mellitin), and TRPV (vanilloid) subfamilies, which varies significantly in their selectivity and mode of activation (5). The understanding of the function, gating, regulation, and structure assembly of the TRP family is developing rapidly. Initially, it was demonstrated that the Drosophila TRP and TRPL members form heteromultimeric channels associated in a supramolecular signaling complex with specific receptors and regulators (6). Moreover, it has been identified that there are many channel compositions within the TRPC family, e.g. TRPC1/3, TRPC1/5, TRPC4/5, and TRPC3/6/7 (7–9). Within the TRPV family, the oligomeric structure of TRPV1 was studied by biochemical cross-linking, and the predominant existence of tetramers was suggested (10). More recently, it has been reported that TRPV5 and TRPV6 form homo- or heterotetramers in order to generate a pleiotropic set of functional channels with different Ca\(^{2+}\) transport (11–13). TRPV5 and TRPV6 share 75% sequence homology at the amino acid level (11–13) and display several similar functional properties, including the permeation profile for monovalent and divalent cations (14) and regulation by calciotropic hormones (15–21). However, detailed sequence comparison of the N- and C-tails of the TRPV5 and TRPV6 channels reveals significant differences, which may account for the unique electrophysiological properties including differences of inactivation, kinetic properties, and affinity for the blocker ruthenium red between these two homologous channels (22).

A considerable amount of information in channel subunit assembly has been accumulated by studies on voltage-gated K\(^{+}\) (K\(_{\text{v}}\)) channels that are structurally related to the TRP channels. Previous studies indicated that the subfamily of Shaker-related K\(_{\text{v}}\) channels possesses a T1 domain in the N-tail, which confers subfamily specificity and inter-subunit assembly (23). This highly conserved T1 domain has been shown to spontaneously form tetramers in the absence of transmembrane sequences (24, 25). Crystallization of this domain further verified the reported biochemical and functional data and implicated that T1 is also involved in channel gating (25, 26). To date, no information is available about assembly domains in TRP proteins. Because TRPV5 shares common structural features with K\(_{\text{v}}\) channels, we hypothesized that there is at least one assembly signal within this channel.

The aim of the present study was to identify the regions in TRPV5 involved in channel assembly. By using different experimental approaches, including pull-down, co-immunoprecipitation, patch clamp, and immunoocytochemical analysis, two

\(^1\) The abbreviations used are: TRP, transient receptor potential; GST, glutathione S-transferase; HA, hemagglutinin; TRPC, TRP canonical; TRPV, TRP vanilloid; HA, hemagglutinin; RT, room temperature; PMSF, phenylmethylsulfonyl fluoride; NFDM, non-fat dried milk; MES, 2-(N-morpholino)ethanesulfonic acid; ANK, ankyrin.
critical domains in the N-tail and C-tail were identified to be 595–600) were obtained by N-tail (deleted amino acids 64–76) or the C-tail (deleted amino acids 87–99) of the pT7Ts vector (21). Deletion mutants of the assembly domain in the pT7Ts vector (21). Deletion mutants were verified by sequence analysis. The predicted potential of the N- and C-tails of TRPV5 were truncated proteins in which the N-tail (TRPV5 N-tail) or C-tail (TRPV5 C-tail) were deleted were obtained by PCR and cloned into the pCIneo/IRE-SP-GFP vector and subcloned into the pTTVs vector (21). Deletion mutants of the assembly domain in the N-tail (deleted amino acids 64–76) or the C-tail (deleted amino acids 85–96) were obtained by in vitro mutagenesis and subcloned into the pTTTs vector. (For N-tail deletion, forward primer 5'-ATGACTCAAGGAGGTGAGGAGAGACCGCGGCCTGCCCCACCTCAGGACCATGCGGAGCCGGG-3' and reverse primer 5'-TCAGAAGATGGTAGACTCTC-3' were used. For FLAG-tagged TRPV5 C-tail, forward primer 5'-ATGTTCAAGCAAGGGGCAGGAGCTGACGTCGAC-3' and reverse primer 5'-TCAGAAGATGGTAGACTCTC-3' were used. For HA-tagged TRPV5 C-tail, forward primer 5'-ATGACTCAAGGAGGTGAGGAGAGACCGCGGCCTGCCCCACCTCAGGACCATGCGGAGCCGGG-3' and reverse primer 5'-TCAGAAGATGGTAGACTCTC-3' were used. For FLAG-tagged TRPV5 C-tail, forward primer 5'-ATGTTCAAGCAAGGGGCAGGAGCTGACGTCGAC-3' and reverse primer 5'-TCAGAAGATGGTAGACTCTC-3' were used.) pT7Ts constructs were linearized, and cRNA was synthesized in vitro as described previously. (21) All constructs were verified by sequence analysis.

Electrophysiology—X. laevis oocytes were injected with 5 ng of cRNA of TRPV5 N-tail or C-tail RNA or cRNA transcribed from the deletion mutants. Oocytes were subsequently homogenized with 150 μl of solubilization buffer (20 mM Tris (pH 8.0), 10 mM EDTA, 0.5% (v/v) Nonidet P-40, 10 mM leupeptin, 10 mM pepstatin) and incubated on ice for 1 h and centrifuged at 16,000 × g for 1 h at 4 °C. The soluble proteins were added to antibody-bound beads in sucrose buffer (100 mM NaCl, 20 mM Tris (pH 8.0), 5% (v/v) sucrose, 10 mM leupeptin, 10 μg/ml pepstatin) and incubated overnight at 4 °C. Subsequently, the beads were washed with IPP100 and proteins were eluted in Laemmli as described previously (21). The proteins were transferred to an enhanced chemiluminescence system (Pierce).

Immunocytochemistry—X. laevis oocytes were injected with 5 ng of cRNA of TRPV5, TRPV5 N-tail, or TRPV5 C-tail RNA. Ca2+ uptake was determined 2 days after injection as described previously (2). Plasma Membrane Isolation—X. laevis oocytes were injected with 5 ng of HA-TRPV5 cRNA only or co-injected with 10 ng of TRPV5 N, TRPV5 C or TRPV5 NAC RNA. Ca2+ uptake was determined 2 days after injection as described previously (2).

RESULTS

Pull-down Assay of N-tail and C-tail of TRPV5—The role of TRPV5 N-tail and C-tail in the channel assembly was initially examined using pull-down assays. TRPV5 N-tail and C-tail were expressed as GST fusion proteins and subsequently tested for their interaction in vitro translated full-length TRPV5 or the TRPV5 N-tail and C-tail. As depicted in Fig. 1A, the GST-fused N-tail and C-tail associated with in vitro translated 35S)methionine-TRPV5. Moreover, pull-down analysis demonstrated N-tail-Tail-N (Fig. 1B) and C-tail-C-tail interactions (Fig. 1C). To investigate whether the N-tail preferentially interacts with the N- or C-tail, the GST-N-tail was incubated with in vitro translated N- and C-tail simultaneously. This

Assembly of TRPV5 Channels

DNA Constructs and cRNA Synthesis—The N- and C-tails of TRPV5 were amplified and tagged with an HA or FLAG tag, respectively, by the use of PCR on the full-length cDNA of TRPV5, and were subsequently cloned into the pGEX-6p-2 (Amersham Biosciences) vector and the pTTVs Xenopus laevis oocytes expression vector (27). (For HA-tagged TRPV5 N-tail, forward primer 5'-ATGACTCAACATGAGGACGTCCGCACCATGACCCGGTGCTCTCCACCCACCAAG-3' and reverse primer 5'-TTTAAACGCGGTGGTGGCCACAAC-3' were used.) TRPV5 truncated proteins in which the N-tail (TRPV5 N-tail) or C-tail (TRPV5 C-tail) were deleted were obtained by PCR and cloned into the pCIneo/IRE-SP-GFP vector and subcloned into the pTTTs vector (21). Deletion mutants of the assembly domain in the N-tail (deleted amino acids 64–76) or the C-tail (deleted amino acids 85–96) were obtained by in vitro mutagenesis and subcloned into the pTTTs vector. (For N-tail deletion, forward primer 5'-CTCGCTTCCTGTGG-3' and reverse primer 5'-CGCCGCTGCGTGGCCGCGG-3' were used.) pT7Ts constructs were linearized, and cRNA was synthesized in vitro as described previously (21). All constructs were verified by sequence analysis.

Electrophysiology—X. laevis oocytes were injected with 5 ng of cRNA of HA-tagged TRPV5 or co-injected with 5 ng of cRNA of HA-tagged TRPV5 and 10 ng of nontagged TRPV5 mutants (TRPV5 ΔN, TRPV5 ΔNAC, and TRPV5ΔNΔC), respectively. Immunocytochemistry was performed as described previously (30, 31).

Immunocytochemistry—X. laevis oocytes were injected with 5 ng of cRNA of HA-TRPV5 N-tail or C-tail RNA or cRNA transcribed from the deletion mutants. Oocytes were subsequently homogenized with 150 μl of solubilization buffer (20 mM Tris (pH 8.0), 10% (v/v) glycerol, 5 mM EDTA, 0.5% (v/v) Nonidet P-40, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin) and incubated on ice for 1 h and centrifuged at 16,000 × g for 1 h at 4 °C. The soluble proteins were added to antibody-bound beads in sucrose buffer (100 mM NaCl, 20 mM Tris (pH 8.0), 5% (v/v) sucrose, 10 mM leupeptin, 10 μg/ml pepstatin) and incubated overnight at 4 °C. Subsequently, the beads were washed with IPP100, and proteins were eluted in Laemmli as described previously (21). The proteins were transferred to an enhanced chemiluminescence system (Pierce).

Co-immunoprecipitation—Twenty-microliter equivalents of protein A-coupled agarose beads (Amersham Biosciences) were preincubated for 3 h at RT with 2 μl of monocular anti-HA antibody (Sigma) in 0.7 ml of IPP500 (500 mM NaCl, 10 mM Tris (pH 8.0), 0.1% (v/v) Nonidet P-40, 0.1% (v/v) Tween 20, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin) and 0.1% (v/v) bovine serum albumin. The beads were washed three times with IPP100 (100 mM NaCl, 10 mM Tris (pH 8.0), 0.1% (v/v) Nonidet P-40, 0.1% (v/v) Tween 20, 1 mM PMSF, 10 μg/ml leupeptin, 10 μg/ml pepstatin). These experiments were performed using an enhanced chemiluminescence system (Pierce).
pull-down experiment indicated that the GST-N-tail preferentially binds the in vitro translated N-tail (Fig. 1D). All interactions were specific, as no interaction with GST alone was observed.

Co-immunoprecipitation of N-tail and C-tail of TRPV5—The findings from the pull-down experiments suggested that the N-tail and C-tail of TRPV5 contributed to channel assembly. Therefore, we tested whether the N-tail and C-tail of TRPV5 can be co-immunoprecipitated. First, immunoblot analysis confirmed expression of both tails that were specifically detected by the applied anti-HA or anti-FLAG antibody, respectively. The anti-HA antibody specifically detected the HA-N-tail in total lysates from oocytes co-expressing HA-N-tail and FLAG-C-tail (Fig. 2A). The HA- and FLAG-tagged N-tails (Fig. 2B), HA- and FLAG-tagged C-tails (Fig. 2C), or HA-N-tail with FLAG-C-tail of TRPV5 (Fig. 2D) were co-expressed and subsequently immunoprecipitated using the anti-HA antibody. Immunooblots containing the immune complexes were probed with the anti-FLAG antibody. Co-immunoprecipitations were observed between HA- and FLAG-tagged N-tails (B), HA- and FLAG-tagged C-tails (C), and HA-tagged N-tail and FLAG-tagged C-tail of TRPV5 (D). No binding to GST alone was detected.

Functional Analysis of TRPV5 Channel Assembly—To further elucidate the functional consequences of the observed interactions between N-N tail, N-C tail, and C-C tail, the effect of co-expressing TRPV5 wild-type and truncated proteins (TRPV5ΔN, TRPV5ΔC, or TRPV5ΔNΔC) on TRPV5 activity at the plasma membrane was determined in X. laevis oocytes and HEK293 cells. First, oocytes were injected with HA-TRPV5 only or co-injected with TRPV5ΔN, TRPV5ΔC, or TRPV5ΔNΔC cRNA. Expression of TRPV5 resulted in ~3.5-fold increase of 45Ca2+ influx compared with noninjected oocytes (Fig. 3A). Co-expression of TRPV5ΔN significantly decreased the TRPV5-mediated Ca2+ influx compared with TRPV5 alone, whereas co-expression of TRPV5ΔC completely inhibited Ca2+ influx to a level that was indistinguishable from noninjected oocytes. In contrast, co-expression of TRPV5ΔNΔC had no significant effect on TRPV5 channel activity (Fig. 3A). 45Ca2+ uptake in oocytes expressing only TRPV5ΔN, TRPV5ΔC, or TRPV5ΔNΔC was not different from noninjected oocytes (data not shown). Second, patch clamp analysis was performed to investigate the electrophysiological properties of channel complexes consisting of wild-type and TRPV5 truncated proteins. Truncated channels, including TRPV5ΔN, TRPV5ΔC, and TRPV5ΔNΔC, were co-expressed with wild-type TRPV5 in HEK293 cells. As shown in Fig. 3B, HEK293 cells expressing TRPV5ΔN, TRPV5ΔC or TRPV5ΔNΔC did not yield any Na+ currents and were not different from nontransfected cells (data not shown). Remarkably, HEK293 cells co-expressing wild-type TRPV5 and...
TRPV5ΔN or TRPV5ΔC displayed significantly reduced Na⁺ currents compared with wild-type TRPV5 alone. Inversely, Na⁺ currents measured in HEK293 cells co-expressing wild-type TRPV5 and TRPV5ΔNΔC were not significantly affected, indicating that it is unlikely that the transmembrane domains of TRPV5 are critical determinants for subunit assembly.

**FIG. 3.** Functional analysis of TRPV5 channel assembly. A, ⁴⁵Ca²⁺ uptake was measured in oocytes injected with TRPV5 cRNA only or co-injected with TRPV5ΔN, TRPV5ΔC, or TRPV5ΔNΔC cRNA. B, HEK293 cells were transfected with wild-type TRPV5 or TRPV5 truncated proteins, including TRPV5ΔN, TRPV5ΔC, or TRPV5ΔNΔC, or co-transfected with wild-type TRPV5 and TRPV5ΔN, TRPV5ΔC, or TRPV5ΔNΔC, and subsequently analyzed by patch clamp analysis. * indicates significance compared with TRPV5 transfected cells, p < 0.05. The Na⁺ currents in HEK293 cells transfected with TRPV5ΔN, TRPV5ΔC, or TRPV5ΔNΔC only were not significant different from nontransfected cells (data not shown). HA-TRPV5 and truncated proteins were co-injected in X. laevis oocytes. To isolate plasma membrane fractions follicle membranes were removed, and plasma membranes were coated with silica. Plasma membrane fractions (C) or total membranes (D) were isolated, blotted, and analyzed for TRPV5 expression using monoclonal anti-HA antibody. NI, noninjected; NT, nontransfected.

**TRPV5 Routing Is Disturbed by Mutant Channel Assembly**—In order to understand the molecular mechanism underlying the dominant-negative effect of TRPV5 truncated proteins (TRPV5ΔN and TRPV5ΔC) on wild-type TRPV5 channel activity, a biochemical and immunocytochemical approach was combined. The expression of HA-TRPV5 was determined by analyzing plasma membrane and total membrane fractions of oocytes expressing TRPV5 alone or in combination with the truncated proteins. A distinct band at a molecular mass of ~85 kDa corresponding to the glycosylated form of TRPV5 was observed in plasma membrane fractions of TRPV5-expressing oocytes (Fig. 3C). Most importantly, wild-type TRPV5 expression was significantly reduced or absent in plasma membrane fractions of oocytes co-expressing TRPV5ΔN or TRPV5ΔC, respectively. However, co-injection of TRPV5ΔNΔC had no effect on the plasma membrane localization of TRPV5 (Fig. 3C). Most importantly, total membrane fractions showed that TRPV5 was expressed to a similar extent in the absence and presence of the TRPV5 truncated proteins (Fig. 3D). These results were verified by immunocytochemistry to detect TRPV5 localization on the plasma membrane. In contrast to noninjected oocytes (Fig. 4A), wild-type TRPV5 cRNA-injected oocytes displayed a strong immunopositive labeling along the plasma membrane.

**FIG. 4.** TRPV5ΔN and TRPV5ΔC expression impaired TRPV5 plasma membrane trafficking. X. laevis oocytes were injected with cRNA of HA-tagged wild-type TRPV5 or co-injected with cRNA of HA-tagged wild-type TRPV5 and cRNA of nontagged TRPV5ΔN, TRPV5ΔC, or TRPV5ΔNΔC. Immunocytochemistry was performed on X. laevis oocytes to investigate the effect of TRPV5 truncated proteins on TRPV5 localization. Oocytes injected with wild-type TRPV5 showed predominantly immunopositive TRPV5 staining along the plasma membrane (B), whereas TRPV5 staining in oocytes co-expressing TRPV5ΔN ΔC (D) or TRPV5ΔC (E) was not localized at the plasma membrane but accumulated intracellularly. In addition, oocytes co-injected with wild-type TRPV5 and TRPV5ΔNΔC displayed immunopositive staining of the channel along the plasma membrane (C). As a negative control noninjected (NI) oocytes were treated in a similar way and were devoid of any staining (A).
whereas the cytoplasm was only faintly stained (Fig. 4B). Most importantly, plasma membrane staining of TRPV5 was significantly decreased in oocytes co-expressing HA-TRPV5 and TRPV5ΔN and to a lesser extent HA-TRPV5 and TRPV5ΔC (Fig. 4, D and E). A strong immunopositive staining was, however, present along the plasma membrane of oocytes co-injected with HA-TRPV5 and TRPV5ΔN/ΔC (Fig. 4C). The localization of TRPV5 in the presence of TRPV5ΔN or TRPV5ΔC was predominantly intracellular. Together, these results indicated that the trafficking of HA-TRPV5 from cytosol toward the plasma membrane was disturbed by assembly with TRPV5ΔN or TRPV5ΔC, but not with TRPV5ΔNΔC, explaining the reduced channel activity. Most importantly, immunoblot analysis demonstrated that the total expression of TRPV5 was equal under all conditions (Fig. 3D).

Identification of the Assembly Signal in the N- and C-tail of TRPV5—To gather more detailed information regarding structural requirements for channel subunit assembly of TRPV5, a series of deletion mutants of the N- and C-tails were constructed (Fig. 5). Truncated forms of TRPV5 N-tail and C-tail were expressed as GST fusion proteins and subsequently tested for their interaction with in vitro translated [35S]methionine TRPV5 N-tail (left) or C-tail (right). Interaction of the N-tail or C-tail with the GST fusion proteins was determined by immunoblotting. The assembly domain in the N-tail was localized between residues 64 and 77 (left), whereas the interaction region in the C-tail (right) was observed between residues 596 and 601. GST was used as a negative control in the pull-down experiments. These assembly domains identified in the N- and C-tail are functionally conserved within TRPV5 and TRPV6 members as indicated by the alignments.

![Fig. 5. Mapping of assembly domains in TRPV5.](image)

**Identification of the Assembly Signal in the N- and C-tail of TRPV5**

- As shown in Fig. 5B, whereas the cytoplasm was only faintly stained (Fig. 4B). Most importantly, plasma membrane staining of TRPV5 was significantly decreased in oocytes co-expressing HA-TRPV5 and TRPV5ΔN and to a lesser extent HA-TRPV5 and TRPV5ΔC (Fig. 4, D and E). A strong immunopositive staining was, however, present along the plasma membrane of oocytes co-injected with HA-TRPV5 and TRPV5ΔN/ΔC (Fig. 4C). The localization of TRPV5 in the presence of TRPV5ΔN or TRPV5ΔC was predominantly intracellular. Together, these results indicated that the trafficking of HA-TRPV5 from cytosol toward the plasma membrane was disturbed by assembly with TRPV5ΔN or TRPV5ΔC, but not with TRPV5ΔNΔC, explaining the reduced channel activity. Most importantly, immunoblot analysis demonstrated that the total expression of TRPV5 was equal under all conditions (Fig. 3D).

- **Identification of the Assembly Signal in the N- and C-tail of TRPV5**—To gather more detailed information regarding structural requirements for channel subunit assembly of TRPV5, a series of deletion mutants of the N- and C-tails were constructed (Fig. 5). Truncated forms of TRPV5 N-tail and C-tail were expressed as GST fusion proteins and subsequently tested for their interaction with in vitro translated [35S]methionine TRPV5 N-tail (left) or C-tail (right). Interaction of the N-tail or C-tail with the GST fusion proteins was determined by immunoblotting. The assembly domain in the N-tail was localized between residues 64 and 77 (left), whereas the interaction region in the C-tail (right) was observed between residues 596 and 601. GST was used as a negative control in the pull-down experiments. These assembly domains identified in the N- and C-tail are functionally conserved within TRPV5 and TRPV6 members as indicated by the alignments.

- **Identification of the Assembly Signal in the N- and C-tail of TRPV5**—To gather more detailed information regarding structural requirements for channel subunit assembly of TRPV5, a series of deletion mutants of the N- and C-tails were constructed (Fig. 5). Truncated forms of TRPV5 N-tail and C-tail were expressed as GST fusion proteins and subsequently tested for their interaction with in vitro translated [35S]methionine TRPV5 N-tail (left) or C-tail (right). Interaction of the N-tail or C-tail with the GST fusion proteins was determined by immunoblotting. The assembly domain in the N-tail was localized between residues 64 and 77 (left), whereas the interaction region in the C-tail (right) was observed between residues 596 and 601. GST was used as a negative control in the pull-down experiments. These assembly domains identified in the N- and C-tail are functionally conserved within TRPV5 and TRPV6 members as indicated by the alignments.

- **Identification of the Assembly Signal in the N- and C-tail of TRPV5**—To gather more detailed information regarding structural requirements for channel subunit assembly of TRPV5, a series of deletion mutants of the N- and C-tails were constructed (Fig. 5). Truncated forms of TRPV5 N-tail and C-tail were expressed as GST fusion proteins and subsequently tested for their interaction with in vitro translated [35S]methionine TRPV5 N-tail (left) or C-tail (right). Interaction of the N-tail or C-tail with the GST fusion proteins was determined by immunoblotting. The assembly domain in the N-tail was localized between residues 64 and 77 (left), whereas the interaction region in the C-tail (right) was observed between residues 596 and 601. GST was used as a negative control in the pull-down experiments. These assembly domains identified in the N- and C-tail are functionally conserved within TRPV5 and TRPV6 members as indicated by the alignments.
DISCUSSION

In the present study molecular determinants were identified in TRPV5 that play a key role in the formation of the functional channel complex. Using a combined approach of several independent methods, we conclude that at least two regions in the cytosolic tails of TRPV5 are important for channel assembly and subsequent routing to the plasma membrane. This conclusion is based on the following: (i) pull-down assays and co-immunoprecipitations that demonstrated physical interactions between the N-tail/N-tail, N-tail/C-tail, and C-tail/C-tail of TRPV5; (ii) \(^{45}\)Ca\(^{2+}\) uptake measurements in oocytes and patch clamp analysis in HEK293 cells that showed the dominant-negative nature of TRPV5 truncated channel subunits, lacking the N-tail or C-tail, on TRPV5 wild-type channel activity; (iii) plasma membrane fractions and immunocytochemical analysis that demonstrated a disturbed trafficking of TRPV5 in oocytes co-expressing TRPV5 and TRPV6 toward the plasma membrane explaining the molecular mechanism of the reduced channel activity; (iv) mapping assays that revealed two critical assembly domains located at positions 64–77 in the N-tail and 596–601 in the C-tail of TRPV5; (v) deletion of these assembly domains in the N-tail and C-tail that abolished the interaction between the tails pointing to an important role of these regions in channel assembly.

By analogy with other cation channel subunits consisting of six transmembrane-spanning domains, TRP members are believed to assemble into homo- or heterotetrameric complexes. These complexes have been verified by classical methods such as co-immunoprecipitation, cross-linking analysis, or functional assays applying dominant-negative pore mutants (7–10, 32). Recently, we demonstrated a (hetero)tetrameric stoichiometry for TRPV5 and TRPV6 (32). Heterotetrameric TRPV5/6 proteins displayed properties that, depending on the subunit configuration, are intermediate between TRPV5 and TRPV6. Exchanging TRPV5 for TRPV6 subunits in a channel tetramer has major effects on Ba\(^{2+}\) permeability, Ca\(^{2+}\)-dependent inactivation, and the block by ruthenium red (32). In this way, Ca\(^{2+}\)-transporting epithelia co-expressing TRPV5 and TRPV6 may be able to generate a pleiotropic set of functional heterotetrameric channels. To date, little information is available about assembly domains in TRP proteins.

Our understanding of the architecture of functional TRP channels reflects only the end point of a complex and the poorly understood assembly pathway. Current research is beginning to elucidate the intermediate steps between the synthesis and insertion of individual subunits into the membrane. Our data indicated N-tail-N-tail and C-tail-C-tail interactions within the TRPV5 channel complexes. In addition, co-immunoprecipitations demonstrated that assembly of TRPV5 subunits occurred not only within N-tails or C-tails but also between the N-tail and C-tail. In order to further explore the crucial domains in the N-tail and C-tail for TRPV5 channel assembly, we constructed a series of truncated deletion mutants. Pull-down analysis indicated that a short peptide stretch at amino acids 64–77 is necessary for N-tail/N-tail interaction, whereas the critical region for C-tail/C-tail interaction is located between amino acids 596 and 601. Furthermore, we demonstrated by pull-down assays and co-immunoprecipitations that N- and C-tail mutants lacking these assembly domains are not able to interact with the TRPV5 tails. These findings indicated that the interaction between TRPV5 channel subunits depends on assembly signals present in the N-tail and C-tail.

It is likely that assembly also occurs within the N-tail and C-tail of TRPV6 because TRPV5 and TRPV6 share more than
75% homology at the amino acid level, raising the possibility that both N-tail and C-tail assembled together in order to form functional heterotetrameric channel complexes of TRPV5 and TRPV6 (22). Recently, Niemeyer and co-workers (33) demonstrated that the third ANK repeat within the N-tail of TRPV6 is critical for TRPV6 subunit assembly. This repeat initiates a molecular zipperping process that proceeds past the fifth ANK repeat and thereby creates an intracellular anchor necessary for functional subunit assembly. In addition, deletion of this region prevented TRPV6-TRPV6 self-association and generation of functional channels (33). Pull-down mapping experiments and co-immunoprecipitations in our study identified an assembly domain in the N-tail of TRPV5 that is located more upstream, overlapping with the first ANK repeat. The most significant binding was found between the N-terminal regions 64–77. However, a slightly stronger binding signal was observed with the truncated protein at position 162 that contains the first three ANK repeats (33). These findings may suggest that the ANK repeat identified in TRPV6 could also be involved in TRPV5 channel assembly. ANK repeats have been frequently implicated in protein-protein interactions. In addition, deletion of this molecular zippering process that proceeds past the fifth ANK repeat significantly reduced in HEK293 cells co-expressing wild-type TRPV5 and TRPV5 channel activity was not significantly affected in cells co-expressing wild-type TRPV5 with wild-type TRPV5 and TRPV5 channel complexes that do not reach the plasma membrane. Therefore, assembly of channel subunits is essential for routing of the TRPV5 channel complex and subsequent activity on the plasma membrane.

## References

1. Hoenderop, J. G., Nilius, B., and Bindels, R. J. (2002) *Annu. Rev. Physiol.* 64, 529–549
2. Hoenderop, J. G., van der Kemp, A. W., Hartog, A., van de Graaf, S. F., van Os, C. H., Willems, P. H., and Bindels, R. J. (1999) *J. Biol. Chem.* 274, 8375–8378
3. Hoenderop, J. G., van Leeuwen, J. P., van der Eerden, B. C., Kersten, F. F., van der Kemp, A. W., Merillat, A. M., Waarsingh, J. H., Bossier, B. C., Vollen, V., Hummeler, E., and Bindels, R. J. (2005) *J. Clin. Invest.** 115, 1906–1914
4. Peng, J. B., Chen, X. Z., Berger, U. V., Vassiliev, P. M., Tsukaguchi, H., Brown, K. M., and Hediger, M. A. (2003) *J. Biol. Chem.* 278, 27239–27246
5. Clapham, D. E. (2003) *Nature** 426, 517–524
6. Bahner, M., Sander, P., Paulsen, R., and Huber, A. (2000) *J. Biol. Chem.* 275, 2901–2904
7. Linschinger, B., Balzer-Geldsetzer, M., Baskaran, T., Graier, W. F., Romanin, C., Zhu, M. X., and Groschner, K. (2000) *J. Biol. Chem.* 275, 27799–27805
8. Strubing, C., Krapivinsky, G., Krapivinsky, L., and Clapham, D. E. (2001) *Neuron* 29, 645–655
9. Hofmann, T., Schafer, M., Schultz, G., and Gudermann, T. (2002) *Proc. Natl. Acad. Sci. U. S. A.* 99, 7461–7466
10. Kedari, N., Stabio, T., Lié, J. D., Treanton, J. J., Olah, Z., Iadarola, M. J., and Blumberg, P. M. (2001) *J. Biol. Chem.* 276, 28613–28619
11. Muller, D., Hoenderop, J. G., Meij, I. C., van den Heuvel, I. P., Knoers, N. V., den Hoolander, A. I., Eggert, P., Garcia-Nieto, V., Claverie-Martin, F., and Bindels, R. J. (2000) *Genomics* 67, 48–53
12. Muller, D., Hoenderop, J. G., Merkx, G. F., van Os, C. H., and Bindels, R. J. (2000) *Biochem. Biophys. Res. Commun.* 275, 47–52
13. Weber, K., Erben, R. G., Rump, A., and Adammers, J. (2001) *Biochem. Biophys. Res. Commun.* 289, 1287–1294
14. Nilius, B., Prenen, J., Vennekens, R., Hoenderop, J. G., Bindels, R. J., and Droogmans, G. (2001) *Cell Calcium* 29, 417–428
15. Hoenderop, J. G., Chon, H., Glikka, D., Ebyusen, H. A., Holstege, F. C., St-Arnaud, R., Braam, B., and Bindels, R. J. (2004) *Kidney Int.* 65, 531–539
16. Hoenderop, J. G., Dardenne, O., Van Abel, M., Van Der Kemp, A. W., Van Os, C. H., St-Arnaud, R., and Bindels, R. J. (2002) *FASEB J.* 16, 1399–1406
17. Hoenderop, J. G., Muller, D., Van Der Kemp, A. W., Hartog, A., Suzuki, M., Ishibashi, K., Imai, M., Sweep, F., Willems, P. H., Van Os, C. H., and Bindels, R. J. (2001) *J. Am. Soc. Nephrol.* 12, 1342–1349
18. van Abel, M., Hoenderop, J. G., Dardenne, O., St Arnaud, R., Van Os, C. H., Van Leeuwen, H. J., and Bindels, R. J. (2002) *J. Am. Soc. Nephrol.* 13, 2102–2109
19. van Abel, M., Hoenderop, J. G., van der Kemp, A. W., Van Leeuwen, J. P., and Bindels, R. J. (2003) *Am. J. Physiol.* 285, G78–G85
20. Van Cromphout, S. J., Dewerchin, M., Hoenderop, J. G., Stockmans, I., Van Herck, E., Kato, S., Bindels, R. J., Colen, D., Carmeliet, P., Bouillon, B., and Carmeliet, G. (2002) *Nat. Acad. Sci. U. S. A.* 99, 13324–13329
21. van de Graaf, S. F., Hoenderop, J. G., Glikka, D., Lamers, D., Prenen, J., Rescher, U., Gerke, V., Staab, O., Nilius, B., and Bindels, R. J. (2000) *EMBO J.* 19, 1478–1487
22. Hoenderop, J. G., Vennekens, R., Muller, D., Prenen, J., Droogmans, G., Bindels, R. J., and Nilius, B. (2001) *J. Physiol. (Lond.*)* 537, 747–761
23. Lu, J., Robinson, J. M., Edwards, D., and Deutsch, C. (2001) *Biochemistry* 40, 10934–10946
24. Shen, N. V., and Pfaffinger, P. J. (1995) *Neuron* 14, 625–633
25. Kreusch, A., Pfaffinger, P. J., Stevens, C. F., and Cho, S. (1998) *Nature** 392, 945–948
26. Bixby, K. A., Nanoo, M. H., Shen, N. V., Kreusch, A., Bellamy, H., Pfaffinger, P. J., and Cho, S. (1999) *Nat. Struct. Biol.* 6, 38–43
27. Steinmerer, K., Schwappach, B., Bens, M., Vandelavalle, A., and Jentsch, T. J. (1999) *J. Biol. Chem.* 274, 31172–31177
28. Vennekens, R., Hoenderop, J. G., Prenen, J., Stuiver, M., Willems, P. H., Droogmans, G., Nilius, B., and Bindels, R. J. (2000) *J. Biol. Chem.* 275, 3963–3969
29. Kamsteeg, E. J., Wormald, T. A., Rijse, J. P., Van Os, C. H., and Deen, P. M. (1999) *EMBO J.* 18, 2394–2400
30. Mulders, S. M., Rijse, J. P., Hartog, A., Bindels, R. J., Van Os, C. H., and Deen, Shaker K⁺ channel subfamilies provided valuable structural insights into understanding both channel assembly and functional regulation of the entire channel molecule through conformational changes (25). Most interestingly, previous studies suggested that T1 plays a role not only in channel assembly but also in channel gating and that conformational changes across the buried polar interface between subunits are a crucial part of the gating process (40). Further studies will address the question whether the identified assembly signals in TRPV5 play also a role in channel gating.

In summary, both the N-tail (amino acids 64–77) and C-tail (amino acids 596–601) are critical for TRPV5 channel assembly. Truncated TRPV5 channels at the N- or C-tail oligomerize with wild-type TRPV5 channels leading to the formation of mutant channel complexes that do not reach the plasma membrane. Therefore, assembly of channel subunits is essential for routing of the TRPV5 channel complex and subsequent activity on the plasma membrane.
Assembly of TRPV5 Channels

31. Mulders, S. M., Knoers, N. V., Van Lieburg, A. F., Monnens, L. A., Leumann, E., Wuhl, E., Schober, E., Rijss, J. P., Van Os, C. H., and Deen, P. M. (1997) J. Am. Soc. Nephrol. 8, 242–248
32. Hoenderop, J. G., Voets, T., Hoefs, S., Weidema, F., Prenen, J., Nilius, B., and Bindels, R. J. (2003) EMBO J. 22, 776–785
33. Erler, I., Hirnet, D., Wissenbach, U., Flockerzi, V., and Niemeyer, B. A. (2004) J. Biol. Chem. 279, 34456–34463
34. Curran, A. R., and Engelman, D. M. (2003) Curr. Opin. Struct. Biol. 13, 412–417
35. Li, M., Jan, Y. N., and Jan, L. Y. (1992) Science 257, 1225–1230
36. Shen, N. V., Chen, X., Boyer, M. M., and Pfaffinger, P. J. (1993) Neuron 11, 67–76
37. Covarrubias, M., Wei, A. A., and Salkoff, L. (1991) Neuron 7, 763–773
38. Deal, K. K., Lovinger, D. M., and Tamkun, M. M. (1994) J. Neurosci. 14, 1666–1676
39. Lee, T. E., Philipson, L. H., Kuznetsov, A., and Nelson, D. J. (1994) Biophys. J. 66, 687–693
40. Minor, D. L., Lin, Y. F., Mobley, B. C., Avelar, A., Jan, Y. N., Jan, L. Y., and Berger, J. M. (2000) Cell 102, 657–670