Hypotonic Activation of Short ClC3 Isoform Is Modulated by Direct Interaction between Its Cytosolic C-terminal Tail and Subcortical Actin Filaments

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Short ClC3 isoform (sClC3) functions as a volume-sensitive outwardly rectifying anion channel (VSOAC) in some cell types. In previous studies, we have shown that the hypotonic activation of sClC3 is linked to cell swelling-mediated remodeling of the actin cytoskeleton. In the present study, we have tested the hypothesis that the cytosolic tails of sClC3 bind to actin directly and that binding modulates the hypotonic activation of the channel. Co-sedimentation assays in vitro demonstrated a strong binding between the glutathione S-transferase-fused cytosolic C terminus of sClC3 (GST-sClC3-CT) to filamentous actin (F-actin) but not to globular monomeric actin (G-actin). The GST-fused N terminus (GST-sClC3-NT) exhibited low binding affinity to both G- and F-actin. Co-sedimentation experiments with progressively truncated GST-sClC3-CT indicated that the F-actin binding region is located between amino acids 690 and 760 of sClC3. Two synthetic peptides mapping basic clusters of the cytosolic sClC3-CT (CTP2, isoleucine 716 to leucine 734; and CTP3, proline 688 to proline 709) prevented binding of GST-sClC3-CT to F-actin in vitro. Dialysis into NIH/3T3 cells of these two peptides (but not of synthetic peptide CTP1 (isoleucine 737 to glutamine 748)) reduced the maximal current density by 60 and 38%, respectively. Based on these results, we have concluded that, by direct interaction with subcortical actin filaments, sClC3 contributes to the hypotonic stress-induced VSOACs in NIH/3T3 cells.

Cell volume regulation is a complex and well coordinated process that requires activation of ion transporters. The family of voltage-regulated chloride channels includes at least two hypotonic cell swelling-sensitive channels, ClC2 and sClC3, whose activation contributes to the maintenance of physiological cell volume in Xenopus oocytes and NIH/3T3 cells (1, 2). These channels have different biochemical properties; ClC2 exhibits inward rectification, whereas sClC3 exhibits outward rectification (2–5). However, the mechanisms responsible for activation of ClC2 and sClC3 share some similarities. Both channels form homo- or heterodimers that are required for a functional double-barreled configuration (6–8). The cytosolic N termini play key roles in the activation of both channels, whereas N-terminal truncations produce constitutively active channels in isotonic environment, no longer responsive to hypotonic stress (9, 10). The regulatory role of the N termini may depend on phosphorylation, as activation of protein kinase C (PKC)2 by phorbol esters causes inactivation of both channels in cultured dorsal root ganglion neurons (11), NIH/3T3 cells (12), and pulmonary artery smooth muscle cells (PASMCs) (13). Other protein kinases can also regulate these channels. Phosphorylation by p34cdc2/cyclin B attenuates the ClC2 current and supports progression through the cell cycle of rabbit ClC2 expressed in Xenopus oocytes (14), and in NIH/3T3 cells, HEK293 and NRK-49F cells (15), whereas phosphorylation by the Ca21-dependent protein kinase CaMKII (16) or by the serum- and glucocorticoid-dependent kinase (17) may cause activation of sClC3 in tsA and PASMCs, respectively. Finally, remodeling of the actin cytoskeleton modulates the activation of ClC2 in Xenopus oocytes (18) and of sClC3 in PASMCs (19).

Previous studies have demonstrated a link between PKC and the activation of sClC3. PKC was shown to provide an inhibitory signal required for maintenance of inactive sClC3 in NIH/3T3 cells (12). Studies in PASMCs have expanded on these observations and have corroborated the PKC requirement for activation of VSOAC/sClC3 by showing that dialysis into patched cells of a specific PKC inhibitory peptide activates chloride current under isotonic conditions (20). To understand how PKC regulates VSOAC in intact cells, we have previously carried out experiments that show co-localization of PKC and sClC3 at the surface membrane of resting cells, but dissociation of PKC localization from sClC3 in hypotonically swollen cells (19). We have demonstrated further that translocation of PKC to perinuclear sites coincides with a hypotonic swelling-induced dissociation of peripheral actin filaments (19). Together, these studies establish PKC as a regulatory molecule for the activation of sClC3 current during hypotonic stress. Furthermore, these studies point to the loss of binding sites for active PKC (RACK) in the vicinity of membrane chloride channels as a mechanism for removal of the inhibitory effect of PKC on VSOAC/sClC3. However, these studies do not rule out the possibility that actin may mediate a mechanical PKC-independent

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2 The abbreviations used are: PKC, protein kinase C; PASMC, pulmonary artery smooth muscle cell; GST, glutathione S-transferase; pA/pF, picoamp(s)/picofarad(s); CBS, cystathionine-β-synthase.

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sClC3 Binds to F-actin

Production of GST-fused Cytosolic Tails of Human Short sClC3—The cytosolic N-terminal tail of human short sClC3 (sClC3-NT) was cloned into pGEX-4T-3 plasmid, and fusion peptides were produced in BL21 cells, as described in the Experimental Procedures. GST (control), GST-sClC3-NT, and GST-sClC3-CT were purified by glutathione-Sepharose 4B column chromatography. The quality of unpurified (shown only for GST) (A) and column-purified GST (B), GST-sClC3-NT (C), and GST-sClC3-CT (D) was assessed after Coomassie Brilliant Blue (CBB) staining of total protein in SDS-polyacrylamide gels and after immunoblotting with a GST antibody (GST Ab) and antibodies raised against epitopes of the N terminus (NT Ab) and C terminus (CT Ab) of sClC3.

EXPERIMENTAL PROCEDURES

Production of GST-fused Cytosolic Tails of Human Short sClC3—The cytosolic N-terminal tail of human short sClC3 without the start codon (sClC3-NT, nucleotides 4–207) was amplified by reverse transcription-PCR with primers that included 5′-BamHI and 3′-XhoI cloning sites. The PCR product was ligated into pGEX-4T-3 (Amersham Biosciences). The whole cytosolic C terminus of sClC3 (sClC3-CT, nucleotides 1672–2283) was amplified and cloned into pGEX-4T-3 as well. Cloning was verified by nucleotide sequencing. GST fusion peptides (i.e. GST, GST-sClC3-NT, and GST-sClC3-CT) were expressed in BL21 cells and purified by column chromatography using glutathione-Sepharose 4B matrix and 10 mM reduced glutathione as the elution buffer (Amersham Biosciences). The columns to the right contain the assigned identities (ID) and the predicted molecular sizes (kDa).

Immunoblot analysis verified the molecular size and the purity of the produced peptides for binding experiments.

Binding of GST-sClC3-NT and GST-sClC3-CT to F-actin (Sedimentation Assay)—For this assay, we used an actin-binding protein Biochem kit (catalogue number BK001; Cytoskeleton, Denver, CO) and followed the manufacturer’s protocol. Briefly, F-actin stock solution (23 µM) was mixed with GST-sClC3-NT or -CT fusion peptides that were preclarified by centrifugation (200,000 x g for 1 h at 4 °C). The reactions were incubated at 22 °C for 1 h, and after a second centrifugation (150,000 x g for 1 h at 22 °C), pellets were resuspended in Laemmli buffer and protein was resolved by SDS-PAGE. Gels were stained with Sypro Ruby, a protein-specific stain with high sensitivity (1–10 ng of total protein) and a linear range that extends over three orders of magnitude (Bio-Rad). For Western blot analysis, proteins were transferred onto nitrocellulose. Membranes were probed with polyclonal antibodies raised against N-terminal (amino acids 1–14, dilution 1:500) or C-terminal (amino acids 670–687, dilution 1:1000) epitopes of sClC3 (21). To assess the results, immunoreactive bands of GST fusion peptides pelleted in the absence of F-actin (negative controls) were subtracted from immunoreactive bands of GST fusion peptides pelleted in complex with F-actin. Other control reactions tested the binding of recombinant GST with F-actin to assess whether association was mediated by the GST-region of the fusion peptides.

Sedimentation Assays with Inhibitory Peptides—Synthetic inhibitory peptides were used to identify the amino acid domains of the cytosolic C terminus of sClC3 that mediate association with F-actin. Sedimentation analyses using progressively truncated C termini demonstrated that domains involved in the binding to F-actin reside in the remote 90 amino acids of the C terminus (Fig. 3). Peptides complementary to three basic amino acid clusters (CTP1, Ile<sup>737</sup>-Gln<sup>748</sup>; CTP2, Ile<sup>716</sup>-Leu<sup>724</sup>; and CTP3, Pro<sup>688</sup>-Pro<sup>709</sup>) were synthesized (Genotide Biosciences, LLC, Lawrence, KS) and added into binding reactions at concentrations ranging between 3 and 100 µM. The sedimentation assay was carried out and quantified as described in the previous section.

Binding of GST-sClC3-NT and -CT to G-actin (Pulldown Assay)—G-actin solutions with concentrations ranging from 0.3 to 30 µM were incubated at room temperature with fixed concentrations of GST, GST-sClC3-NT, or GST-sClC3-CT.
sClC3 binds to F-actin

FIGURE 4. The cytosolic sClC3-CT binds to G-actin in vitro. GST (A1–A3), GST-sClC3-NT (B1–B3), and GST-sClC3-CT (C1–C3) were immobilized on glutathione-Sepharose beads and incubated with increasing concentrations of G-actin (0.3–30 μM). Protein from reaction supernatants (SN, A1–C1) and pellets (PT, A2–C2 and A3–C3) was resolved by SDS-PAGE and stained with Sypro Ruby (A1–C1 and A2–C2) or transferred onto nitrocellulose and probed with a polyclonal actin antibody (A3–C3). Peptides immobilized on glutathione-Sepharose beads. The beads were washed with phosphate-buffered saline (10 mM Na₂HPO₄, 1.8 mM KH₂PO₄, 2.6 mM KCl, and 137 mM NaCl, pH 7.4) and then mixed with Laemmli buffer and boiled. Proteins were resolved by SDS-PAGE and stained with Sypro Ruby or transferred onto nitrocellulose and probed with a polyclonal actin antibody (A3–C3).

RESULTS

GST-sClC3-NT and GST-sClC3-CT Exhibit Low Binding Affinity for Monomeric G-actin—To evaluate the binding efficiency of the cytosolic tails of sClC3, we first immobilized GST-sClC3-NT or -CT on glutathione-Sepharose beads and then carried out binding experiments with G-actin. The amounts of G-actin that sedimented in the pellets were used for quantification of the G-actin binding to GST-sClC3-NT or -CT. Staining with Sypro Ruby indicated that actin remained primarily in the reaction supernatants (Fig. 4, A1–C1). Staining of pellets with Sypro Ruby revealed GST (Fig. 4, A2), the GST-sClC3-NT doublets (Fig. 4, B2), and GST-sClC3-CT (Fig. 4, C2) as the major proteins associated with glutathione-Sepharose beads. Immunoblotting of the pellets detected measurable amounts of actin.
sClC3 Binds to F-actin

**FIGURE 5. Strong binding of GST-sClC3-CT and weak binding of GST-sClC3-NT to F-actin in vivo.** Co-sedimentation experiments were carried out with an actin-binding protein Biochem kit (Cytoskeleton Inc., Denver, CO) as described under “Experimental Procedures.” GST-sClC3-NT and GST-sClC3-CT were used at a concentration of 10 μM. The binding assays also included α-actinin (positive control (+), lanes 1 in all panels) and GST (negative control (−), lanes 2 in all panels). Total reaction protein was resolved by SDS-PAGE and stained with Sypro Ruby (A and C) or transferred onto nitrocellulose and probed with polyclonal N-terminal (B) or C-terminal (D) antibodies. WB, Western blot. Ab, antibody.

only in the reaction with GST-sClC3-CT (Fig. 4, C3). Because actin did not sediment with GST (Fig. 4, A3), the presence of actin in the pellet is the result of its binding to sClC3-CT.

**GST-sClC3-CT Readily Binds to F-actin—**For these experiments, we first induced actin polymerization prior to testing the binding affinity of the cytosolic N and C termini to F-actin by co-sedimentation. Staining of sedimented proteins with Sypro Ruby demonstrated that the reaction conditions enabled actin polymerization and sedimentation of actin filaments (Fig. 5, A and C, lanes 1, 2, and 4), association of F-actin with α-actinin (positive control, lanes 1), and lack of binding of GST to F-actin (negative control, lanes 2). A band corresponding to GST-sClC3-CT (52 kDa) was also visible in Sypro Ruby-stained gels (Fig. 5C), indicating that GST-sClC3-CT binds to F-actin. However, a band of sedimented GST-sClC3-NT (39 kDa) was indistinguishable due to overlap with the strong actin band (42–44 kDa) (Fig. 5A). To avoid this overlap, we transferred the reaction protein onto nitrocellulose membranes and probed them with sClC3-NT and -CT antibodies. These antibodies exhibited low cross-reactivity with actin but demonstrated strong binding of GST-sClC3-CT and a moderate binding of GST-sClC3-NT to F-actin. Because GST alone did not bind to F-actin, association was mediated by the cytosolic termini of sClC3. The results from these experiments demonstrate that the cytosolic N terminus of sClC3 has low binding affinity for both G- and F-actin; however, the C terminus exhibits higher binding affinity particularly for F-actin.

**Binding to F-actin Is Mediated by a Domain Localized in the Distal C Terminus of sClC3—**To identify the region of sClC3-CT that mediates binding to F-actin, we carried out sedimentation experiments in which F-actin was incubated with progressively truncated C-terminal sClC3 peptides fused to GST (Fig. 2). Whereas the preparations of fusion peptides CT0, CT3, and CT4 were homogeneous, fusion peptides CT1, and to a lesser extent CT2, co-purified with other peptides with smaller molecular sizes (Fig. 6, top panel, lanes 3–6). These by-products were also recognized by the sClC3-CT antibody used in these experiments and may represent shorter fragments of GST-sClC3-CT1 or -CT2. Only the immunoreactive bands of the full-length GST-sClC3-CT peptides (Fig. 6, top bands), but not the bands of the shorter by-products, were taken into account for evaluation of binding to F-actin.

Fusion peptides GST-sClC3-CT1 (lacking 30 C-terminal amino acids) and GST-sClC3-CT2 (lacking 60 C-terminal amino acids) bound to F-actin in a manner similar to the whole C-terminal tail; i.e. GST-sClC3-CT0 (Fig. 6). However, fusion peptides GST-sClC3-CT3 and -CT4, failed to associate with F-actin (Fig. 6). These results indicate that the binding of sClC3 to F-actin is mediated by regions located in the distal 60 amino acids of the cytosolic C terminus of the channel.

**Synthetic Inhibitory Peptides CTP1, CTP2, and CTP3 Differ in Their Ability to Inhibit Binding between GST-sClC3-CT0 and F-actin—**To further characterize the binding of the C terminus of sClC3 with F-actin, we synthesized synthetic peptides, mapping basic clusters within the distal 60 amino acids of sClC3-CT (Fig. 3). We carried out co-sedimentation assays between GST-sClC3-CT0 and F-actin in the presence of increasing concentrations of the inhibitory peptides. CTP1 insignificantly changed the binding of GST-sClC3-CT0 to F-actin (Fig. 7, top panel and bar graph), suggesting that the basic cluster in the sequence mapped by this peptide (i.e. Ile737-Gln748) has little or no effect on the association of sClC3-CT with F-actin. Increas-
ing concentrations of CTP2 (amino acids Ile716–Leu734) reduced the binding of GST-sClC3-CT0 by ~60% at 100 μM (Fig. 7, second panel from the top and bar graph), whereas at the same concentration of CTP3 (amino acids Pro688–Pro709) reduced the GST-sClC3-CT0 binding to F-actin by >90% compared with controls (Fig. 7, third panel from top and bar graph).

These results are consistent with the notion that binding with F-actin is mediated by the C-terminal stretch mapped by CTP2 and CTP3; i.e., between amino acids Pro688 and Leu734 of sClC3.

**Intracellular Dialysis of C-terminal sClC3 Antibody Inhibits Native VSOAC Currents in Cultured NIH/3T3 Cells**—To test whether native sClC3 is responsible for VSOACs in cultured NIH/3T3 cells, we dialyzed cells with C-terminal sClC3 antibody (10 μg/ml) prior to exposure to hypotonic solution. The antibody dialysis caused little change in leak currents under isotonic conditions (1.89 ± 0.43) compared with controls (Fig. 7, first panel from top and bar graph) and GST-sClC3-CT0. CTP1 was the least potent peptide (top blot), CTP2 (second blot from the top), and CTP3 (third blot from the top) exhibited higher inhibitory potency. The average data are depicted in the bar graph. *, p < 0.05 from binding at the inhibitory peptide concentration of 0 μM; n = 3, Ab, antibody.

**FIGURE 7. Synthetic peptides mapping basic amino acid clusters in the C terminus of sClC3 (see Fig. 3) inhibit binding of F-actin to full-length GST-sClC3-CT0.** Peptides were added at increasing concentrations from 3 to 100 μM (blots) into co-sedimentation reaction with fixed concentrations of actin (bottom blot) and GST-sClC3-CT0. CTP1 was the least potent peptide (top blot), CTP2 (second blot from the top), and CTP3 (third blot from the top) exhibited higher inhibitory potency. The average data are depicted in the bar graph. *, p < 0.05 from binding at the inhibitory peptide concentration of 0 μM; n = 3, Ab, antibody.

**FIGURE 8. Intracellular dialysis of an antibody raised against an antigen mapping amino acids 670–687 of the C terminus of sClC3 (but not antigens-preabsorbed antibody) blocks native VSOAC current density.** Membrane currents were induced by 100-ms voltage steps to +80 mV from a holding potential of ~40 mV every 30 s. A, time course of current density in a cell dialyzed with either 10 μg/ml sClC3-CT antibody (Ab) or antigen-preabsorbed antibody (Ag-Ab) under isotonic, hypotonic, and hypertonic conditions. Ctr, control, B, summarized data showing maximum current density at +80 mV under isotonic (Iso) and hypotonic (Hypo) conditions in control (Ctr) cells, cells dialyzed with sClC3-CT antibody (Ab), and antigen-preabsorbed antibody (Ag-Ab). *, p < 0.05 compared with the respective isotonic control; NS, not significant; n, number of cells in each treatment group.

**DISCUSSION**

Although the role of the short isoform of sClC3 as a candidate protein responsible for native VSOACs has been questioned in some cell types (24–26), a variety of new evidence using antisense oligonucleotides, cRNA, and new sClC3 inhib-
sClC3 Binds to F-actin

FIGURE 9. Intracellular dialysis of synthetic peptides CTP2 and CTP3 (but not CTP1) reduces the maximal VSOAC current density. Membrane currents were elicited by voltage pulses to \(-80\) mV from a holding potential of \(-40\) mV every 30 s. Peptides were dialyzed through the patch electrode. A, representative traces of the time course of current density in cells dialyzed with control (Ctr) pipette solution or test peptides CTP1, CTP2, or CTP3 under isotonic, hypotonic, and hypertonic conditions. B, summary of peptide-mediated effects on current density measured at the peak VSOAC activation. C, peptide dialysis does not change the time to half-maximal activation (\(t_{1/2}\)). *, \(p < 0.05\); **, \(p < 0.01\) compared with control; n, number of cells in each treatment group.

Previous studies have shown that dynamic structural rearrangement of the actin cytoskeleton can activate various chloride channels in different cells (18, 19, 33, 34) and can affect the ability of cells to undergo regulatory volume decrease or regulatory volume increase (35). Actin filaments appear to be involved in the activation of at least two Cl\(^{-}\) channels of the CIC family (CIC2 (18) and sClC3 (19)), which may be responsible for regulatory volume decrease. Although both channels are activated during hypotonic cell swelling, the expressed Cl\(^{-}\) currents differ in their biophysical characteristics and in the way they interact with F-actin. CIC2 binds to F-actin with its cytosolic N terminus (18), whereas the sClC3 binding to F-actin occurs primarily through the C terminus. The ability of the C terminus to affect the hypotonic activation of sClC3 is consistent with previous studies in which C termini of other CIC family members modulate channel excitability and gating (36, 37). The regulatory effects of the C termini have been attributed to their 60-amino-acid-long tandem cystathionine-\(\beta\)-synthase (CBS) domains. Although the functions of CBS domains are largely undefined or controversial (38), the importance of the CBS pairs is emphasized by the correlations between mutations of these domains and Cl\(^{-}\) channel-associated hereditary diseases, such as myotonia congenita (mutated CIC1 (39)), idiopathic generalized epilepsy (CIC2 (40)), hypercalciuric nephrolithiasis (CIC5 (41)), autosomal dominant osteopetrosis type II (CIC7 (42)), and Bartter syndrome (CIC-Kb (43)).

The cytosolic C terminus of sClC3 also contains a tandem repeat of CBS-like domains, i.e. a 60-amino-acid-long CBS1 domain (amino acids 596–656) and a 60-amino-acid-long CBS2 domain (amino acids 698–749 (GenBank\(^{TM}\) accession number P51790). Crystal structures of a bacterial homologue predict tightly folded conformation of CBS pairs composed of two symmetrical CBS motifs arranged in an anti-parallel manner (38, 44). However, a variety of experimental methods have revealed only a partially folded conformation and a larger than predicted Stokes radius of a water-soluble C terminus of rat sClC3 (45). Sequence alignment of sClC3 demonstrates that the actin binding domain (amino acids 685–750) overlaps with the CBS2 domain (amino acids 697–738) of sClC3. Because folding of some proteins may depend on association with binding partners (46), one can speculate that, by binding to the CBS2 domain, F-actin may induce intramolecular associations for ordered folding of the CBS tandem and for expression of functional sClC3 channels. By interfering with the binding of the C terminus to adjacent F-actin, inhibitory peptides may prevent
normal activation of sClC3. At this time, it can only be speculated whether the interactions between sClC3 and F-actin in cultured cells has functional significance at the organ or tissue level, as no animal or human pathological condition has yet been linked to abnormalities of sClC3. However, disruption of the CICN3 gene in mice causes severe retinal degeneration, loss of hippocampus and ileal mucosa (26), and development of a phenotype similar to human neuronal ceroid lipofuscinosis (47). Because interference with the binding of sClC3 to adjacent actin filaments results in activation-deficient sClC3 channels, these intermolecular interactions could underlie developmental abnormalities observed in mice.

In conclusion, our results demonstrate a direct association of the cytosolic C terminus of human short sClC3 with subcortical actin filaments. Interaction between the two proteins is required for the activation of sClC3 by hypotonic cell swelling, whereas inhibition of this interaction produces channels with limited capacity for hypotonic activation. It remains to be tested whether mutations in the actin binding region or CBS2 domain render actin binding and/or activation-deficient sClC3 channels responsible for phenotypic and/or functional abnormalities.

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