Cortactin Controls Surface Expression of the Voltage-gated Potassium Channel Kv10.1

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Background: Kv10.1 is a potassium channel expressed in the brain and important for non-neural tumorigenesis.

Results: An interaction between the C terminus of Kv10.1 and the proline-rich domain of cortactin stabilizes the channel at the plasma membrane.

Conclusion: Cortactin interacts with Kv10.1 and controls surface expression of the channel.

Significance: Our findings provide a functional and mechanistic link between the functions of two oncology-relevant proteins.

Kv10.1 is a voltage-gated potassium channel aberrantly expressed in many cases of cancer, and participates in cancer initiation and tumor progression. Its action as an oncoprotein can be inhibited by a functional monoclonal antibody, indicating a role for channels located at the plasma membrane, accessible to the antibody. Cortactin is an actin-interacting protein implicated in cytoskeletal architecture and often amplified in several types of cancer. In this study, we describe a physical and functional interaction between cortactin and Kv10.1. Binding of these two proteins occurs between the C terminus of Kv10.1 and the proline-rich domain of cortactin, regions targeted by many post-translational modifications. This interaction is specific for Kv10.1 and does not occur with Kv10.2. Cortactin controls the abundance of Kv10.1 at the plasma membrane and is required for functional expression of Kv10.1 channels.

Actin cytoskeleton remodeling is crucial for many cellular processes such as migration, invasion, endocytosis, or cell cycle progression. Cortactin (CTTN) therefore plays a central role in these processes, because it interacts with the cortical actin network and is able to alter it (see for example, Ref. 1). To fulfill this role, CTTN requires the interplay of several functional domains and different binding partners as well as post-translational modifications. CTTN binds F-actin through the actin binding repeats (1) and regulates its polymerization via binding and activation of the Arp2/3 complex at the CTTN N-terminal acidic domain (2, 3). This region is followed by an α-helical domain including a calpain cleavage site and a proline-rich region, which harbors multiple tyrosine, threonine, and serine phosphorylation sites. Many kinases, including Src, are reported to act on these sites to control CTTN function in response to intracellular signaling cascades. Therefore, CTTN is thought to act as a key regulator transmitting kinase signaling toward actin remodeling (4). At its extreme C terminus, CTTN possesses a Src homology 3 domain (SH3), where binding of several interacting partners is observed, linking CTTN to several additional cell signaling pathways (1).

Lately, CTTN has been reported to affect the voltage-gated potassium channel Kv1.2 and the large conductance calcium- and voltage-activated potassium channel, KCa1.1 (BK, KCNMA1), by altering their surface expression or conducting properties, respectively (4–8). It has been proposed that these processes are due to a CTTN-mediated linkage of these ion channels with the cytoskeleton. At least for Kv1.2, this seems to be a phosphorylation-dependent process, which influences receptor-mediated endocytosis of this channel (5, 8).

The voltage-gated potassium channel Kv10.1 has attracted particular interest for its ability to transform cells and its resulting oncological relevance (9, 10). This behavior has been partially attributed to the role of Kv10.1 in cell cycle control downstream of p53 (11). Blocking Kv10.1 in MCF-7 cells arrests them in the G1 phase of the cell cycle and inhibits expression of cell cycle-related genes such as cyclin D1 (CCND1) and E (CCNE) (11–13). On the other hand, Kv10.1 itself is regulated during the cell cycle and it shows altered conductive properties and reduced current in the M phase (14, 15). This regulation process is thought to be induced by alterations of the interaction of the ion channel with the cytoskeleton (15).

In this study, we identified CTTN as an interaction partner of Kv10.1 in vivo and confirmed this with different approaches. Our results clearly show that CTTN expression is essential for the surface expression of Kv10.1 and it dramatically influences the Kv10.1-mediated current by regulating its membrane localization. Given that CTTN is often overexpressed in cancer (being part of the well described 11q3 amplicon (16)) and linked to tumor invasiveness (17), CTTN and Kv10.1 could have a synergistic effect on their transforming properties. CTTN might serve to connect Kv10.1 and central signaling pathways in the cell, for example, during the cell cycle.
**EXPERIMENTAL PROCEDURES**

_Yeast Two-hybrid—_The yeast reporter strain L40 (18) (MATa, trp1, leu2, his3, LYS::lexA-HIS3, URA3::lexA-lacZ) was transformed with pLexN-Kv10.1 by the lithium acetate method and grown on synthetic medium lacking tryptophan. Following further transformation with plasmid pVP16-3-cDNA (postnatal 8 rat brain cDNA library, kindly provided by Nils Brose), double transformants were plated on synthetic medium lacking histidine, leucine, uracil, lysine, and tryptophan and in the presence of the competitive inhibitor of the HIS3 protein 3-amino-1,2,4-triazole. Positive colonies were picked after 4–6 days and tested for β-galactosidase activity using the plate assay. Plasmids from positive clones were rescued and transformed in *Escherichia coli* strain HB101. *E. coli* cells were plated on leucine-lacking medium. Positive clones were further analyzed by yeast retransformation and DNA sequencing.

Expression and Purification of GST-tagged Proteins—Full-length CTNN (accession number NM 005231.3) or fragments N-term (residues 1–329), N-term-H (residues 1–400), HP (residues 360–495) and SH3 (residues 475–551) were cloned into pGEX-4T-1 (GE Healthcare) expression vector to introduce an N-terminal GST tag. Expression was performed in cell lysates in buffer LP (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM MgCl2, 1% Nonidet P-40, protease inhibitors (Roche Applied Science)) with α-bungarotoxin-biotin (α-BTX-biotin) conjugate (Invitrogen) at a final concentration of 0.2 μg/ml for 30 min on ice. To detect membrane and/or internalized Kv10.1-BBS, living cells (HEK-BBS) were incubated in media supplemented with α-BTX-biotin conjugate at a final concentration of 2.5 μg/ml and kept at room temperature for 10 min (membrane) or at 37 °C for 1 h (internalized). For internalized Kv10.1-BBS, cells were washed with ice-cold acid wash buffer (150 mM NaCl, pH 3.0) for 3 min to remove membrane labeling of Kv10.1-BBS. Twice washing with cold PBS removed the residual α-BTX-biotin conjugate. Cells were then harvested and lysed with LP buffer for 20 min on ice. The insoluble fraction was removed by centrifugation at 18,000 × g at 4 °C, and the supernatant was used for ELISA or pull-down experiments. Kv10.1-BBS expressed in *Xenopus* oocytes injected with the corresponding cRNA was processed in the same way as that from HEK-BBS cells.

For pulldown approaches, labeled Kv10.1-BBS was bound to streptavidin-coated magnetic beads (T1, Invitrogen) for at least 30 min at 4 °C. Unbound protein was removed by washing twice with a stringent series of four washing buffers (1, LP; 2, 1% dioxane, 0.5% Nonidet P-40, 300 mM NaCl, 50 mM Tris-HCl, pH 7.4, 5 mM EDTA; 3, 10% dioxane, 0.1% Nonidet P-40, 1 mM NaCl, 50 mM Tris-HCl, pH 7.4, 5 mM EDTA; and 4, TBS).

Quantification of the amount of labeled Kv10.1-BBS was performed by ELISA. After labeling, total cell lysates (30 and 150 μg of protein), were immobilized on streptavidin-coated plates (Pierce) and detected using a C-terminal monoclonal anti-Kv10.1 antibody (Ab33, 5 μg/ml) and a polyclonal anti-mouse secondary antibody (Pierce, 1:500) coupled to peroxidase. ABTS (Invitrogen) was used as a substrate for development and detected in a Wallac Victor2 reader at 405 nm (reference 490 nm). Experiments were performed in duplicate.

Pull-down Experiments—For immunoprecipitation, rat brain lysates (800 μg of total protein) were incubated overnight at 4 °C with 2–5 μg of antibody (anti-Kv10.1 33/62 (21); anti-CTTN (Millipore); or nonspecific mouse IgG κ2b) in buffer A (0.5% Triton X-100, 25 mM Tris-HCl, pH 7.5, 75 mM NaCl, 2.5 mM EDTA and protease inhibitors (Roche Applied Science)). Pull down was performed by adding 20 μl of Protein G/A-coated Sepharose beads (Calbiochem) for 2 h at 4 °C under rotation. After washing five times with buffer A, bound protein was eluted at 70 °C for 10 min using LDS sample buffer containing reducing agent (Invitrogen) and analyzed by SDS-PAGE (Invitrogen) and Western blotting.

To test binding to CTNN (full-length or partial domains), the GST-tagged constructs were incubated with Kv10.1-BBS immobilized on streptavidin-coated beads (Invitrogen), in PBS supplemented with protease inhibitors (Roche Applied Science) overnight at 4 °C. Unbound protein was washed away in five washing steps (PBS) before eluting specifically bound proteins by incubation at 70 °C for 10 min using LDS sample buffer containing reducing agent (Invitrogen). Eluted proteins were analyzed by SDS-PAGE (Invitrogen) and Western blotting.

All pull-down and immunoprecipitation experiments were performed at least three times independently with essentially identical results. The figures show representative examples.

**Electrophysiology—**Recordings on *Xenopus* oocytes were performed as described in Ref. 22 using a Turbo TEC-10CD amplifier (NPI electronics). cRNA was synthesized using the...
configuration on macropatches of the patch clamp amplifier (HEKA Electronics) in the outside-out configuration. Number of channels (N) and single channel currents (i) were determined from the voltage clamp recordings, pipettes had resistances ranging from 0.5 to 1.2 MΩ when filled with 2 M KCl. External solution (NFR) contained 115 mM NaCl, 2.5 mM KCl, 1.8 mM CaCl₂, 10 mM Hepes/NaOH (pH 7.2). NFR was used as external measuring solution. Patches were held at a holding potential of −80 mV. Average currents were determined from at least 300 consecutive identical test pulses to +40 mV, and variance was calculated with PulseTools software (HEKA Electronics). Number of channels (N) and single channel currents (i) were determined from the variance (σ²) versus amplitude (i) plot using Equation 1,

\[ \sigma^2 - \sigma_0^2 = i \cdot l - \frac{i^2}{N} \]  

(Eq. 1)

where σ₀² represents the baseline variance (23).

Data acquisition and analysis was performed with PulsePulseFit, PulseTools (HEKA Electronics), and IgorPro (WaveMetrics). All experiments were performed on oocytes from at least two different animals. The number of individual recordings is indicated in the figures.

Microscopy—HEK293 cells were plated in Nunc chambers (LabTek Chamber Slides 177399) and transfected with BBS-tagged constructs of Kv10.1 using Lipofectamine 2000 (Invitrogen). Labeling was performed 24 h after transfection for 1 h at 37 °C by adding Alexa Fluor 555-BTX conjugate (2 μg/ml, Invitrogen) to the culture medium. After five washing steps with ice-cold PBS, live cell imaging was performed on a Zeiss Axiovert 200M microscope.

For confocal microscopy, HeLa cells were grown on glass coverslips, transfected, and fixed 24 h after transfection in 4% paraformaldehyde in PBS for 10 min at room temperature. Permeabilization was performed using 0.1% Triton X-100 in PBS for 10 min at room temperature. Nonspecific binding sites were blocked with 0.5% gelatin in PBS for 30 min at room temperature. To visualize Kv10.1-BBS, permeabilized cells were treated with Alexa 555-BTX conjugate (0.5 μg/ml, Invitrogen) for 1 h, washed thoroughly with PBS, and mounted with ProLong Gold Antifade Reagent (Invitrogen). To detect focal adhesion kinase (FAK) a mouse anti-FAK (BD Biosciences) antibody was used according to the manufacturer’s instructions, with a secondary antibody conjugated to Alexa Fluor 555 (Invitrogen). Image acquisition was performed using a Zeiss LSM 510 Meta confocal microscope, and analysis was carried out with Fiji.

RESULTS

Kv10.1 and CTTN Interact Directly In Vitro via the Proline-Rich Region of CTTN.—To identify interacting partners of Kv10.1, we performed yeast two-hybrid screens using a rat brain expression library. Full-length Kv10.1 fused to the DNA binding domain of the bacterial LexA transcription factor was used as bait to screen a rat brain cDNA library fused to the activation domain of the VP16 transcription factor. Of 2 million screened clones, eight were positive for the HIS3 marker. After sequencing, one of the clones was found to contain the coding sequence for CTTN. Co-immunoprecipitation studies on native tissue confirmed the interaction of Kv10.1 and CTTN. Immunoprecipitation of Kv10.1 co-precipitated CTTN and, in the converse experiment, antibodies against CTTN precipitated Kv10.1 from rat brain lysates, indicating that physical interaction between CTTN and Kv10.1 occurs in vivo (Fig. 1).

To study the determinants for interaction, we generated tagged proteins for pull-down assays: an N-terminal GST-CTTN fusion protein (purified from bacteria) and an BTX-binding site (BBS)-labeled BTX (BD Biosciences) antibody was used as bait to screen a rat brain cDNA library fused to the activation domain of the VP16 transcription factor. Of 2 million screened clones, eight were positive for the HIS3 marker. After sequencing, one of the clones was found to contain the coding sequence for CTTN. Co-immunoprecipitation studies on native tissue confirmed the interaction of Kv10.1 and CTTN. Immunoprecipitation of Kv10.1 co-precipitated CTTN and, in the converse experiment, antibodies against CTTN precipitated Kv10.1 from rat brain lysates, indicating that physical interaction between CTTN and Kv10.1 occurs in vivo (Fig. 1).

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(residues 1–400), which adds the helical region to N-term; -HP (residues 360–495), corresponding to the helical and proline-rich regions; and -SH3 (residues 475–551), namely the classical SH3 domain of CTTN.

Three of these constructs (N-term, HP, and SH3) were cloned in the pLexN vector and tested in the yeast two-hybrid system for interaction with KV10.1 domains in pVP16–4: N terminus, (residues 1–220), C terminus, (residues 478–963), or full-length KV10.1. We did not detect interactions with the N-term or HP constructs. In this approach, the SH3 domain could not be evaluated because it showed autoactivation activity.

We then tested the ability of KV10.1 to pull down the purified GST-tagged CTTN domains. Only GST-HP clearly showed interaction to KV10.1, whereas no or only a very weak signal was detected in GST-N-term, GST-N-term-H, or GST-SH3 pull-down (Fig. 2D). We were also able to detect binding of the HP region to KV10.1 in a large scale pull-down followed by Coomassie stain (Fig. 2E). This result strongly suggests that the interaction between KV10.1 and CTTN interaction occurs through the proline-rich region of CTTN.

CTTN Influences KV10.1-mediated Current—Having established that CTTN and KV10.1 interact directly, we asked next if there is a functional consequence associated with this interaction. CTTN is known to regulate the current through KV2.1 (by altering its surface expression (5, 8)) and KV1.1 (BK, by changing its interaction with the actin cytoskeletal and thereby the open probability of the channel (6, 7)).

To test whether the KV10.1 current was affected, we manipulated the levels of CTTN in Xenopus laevis oocytes expressing KV10.1. To increase CTTN levels, we injected cRNA encoding human CTTN; to reduce the endogenous CTTN, we injected a specific PTO previously reported to deplete CTTN (8). We then measured the current mediated by KV10.1 channels.

In these experiments, the macroscopic KV10.1 current amplitude depended on the presence of CTTN, but KV10.1 currents in CTTN-depleted or overexpressing oocytes did not differ in kinetics or voltage dependence from those obtained in untreated oocytes. KV10.1 produced slowly activating, outward rectifying currents, which did not inactivate; a slight inward rectification was observed with strong depolarizations compatible with block by intracellular sodium (14) (Fig. 3A). The current-voltage relationships were also unchanged by CTTN depletion or overexpression (Fig. 3B). A defining feature of the KV10.1 current is the dependence of the time constant of activation on the holding potential in such a way that the current activates slower the more negative the prepulse potential (24). This property was also not affected by CTTN depletion or overexpression (Fig. 3C).

Depletion of CTTN by PTO injection reduced or even abolished the KV10.1 current in a dose-dependent manner (Fig. 3D). We subsequently used injections of 5 ng of PTO per oocyte as a
working concentration because it led to a current reduction of more than 80% (to 18 ± 6%) but still allowed quantification of the remaining current (n = 12, p ≤ 0.0001, Fig. 3D). A similar behavior was observed when HEK293 cells expressing Kv10.1 were treated with siRNA against CTTN. Kv10.1 was measured in randomly chosen cells of each group, and the mean current density (pA/pF) was determined. Although the overall current values were not significantly different (210 ± 36 pA/pF in the controls and 179 ± 33 pA/pF in the siRNA-treated cells, p = 0.5), 11 of 28 treated cells (40%) showed current densities below 50 pA/pF, versus 4/23 (17%) in the controls, compatible with the expectation of an effect on current density only in a subset of cells successfully transfected with siRNA.

Increasing CTTN over physiological levels did not further enhance Kv10.1-mediated currents (117 ± 16%, n = 11, p = 0.6, Fig. 3E). This was not due to a saturation of the translation/processing systems of the oocyte, as injection of larger amounts of Kv10.1 cRNA leads to an increase in current amplitudes (not shown). PTO does not hybridize to human CTTN (the best match spans only 8 bases). Therefore, co-injection of the human cRNA should be able to replace the depleted CTTN of oocytes. This replacement restored Kv10.1 current amplitudes to 60 ± 10%, n = 17 (Fig. 3F).

As CTTN is known to play important roles in cytoskeletal organization, it is possible that the effects observed are the result of interference rather than a direct effect on Kv10.1. To test this, we used the Arp2/3 complex inhibitor CK-666 (25). Kv10.1 current amplitude was determined in oocytes incubated with 50 μM CK-666 over 24 h (the entire incubation period after RNA injection). No differences were observed as compared with oocytes treated with vehicle (Fig. 3F), indicating that the effect of CTTN knockdown is independent of its role as an Arp2/3 activator. Taken together, these observations strongly indicate that CTTN regulates the amplitude of Kv10.1-mediated currents.

Regulation by CTTN Is Specific to Kv10.1 and Depends on Its C Terminus—As Kv10.2 was able to bind CTTN in vitro pull-down assays (Fig. 2B), we next examined the possible func-
tional effects of CTTN depletion on KV10.2 currents in Xeno-
pus oocytes. We found that currents evoked by this channel were not significantly affected by depletion of CTTN levels (133 ± 21% of control levels, n = 30, p = 0.2; Fig. 4A). Overexpression of CTTN also did not have an effect on KV10.2-mediated currents (100 ± 17% of control levels, n = 10, p = 0.99; Fig. 4A). The kinetic properties of the macroscopic currents of KV10.2 were also unaffected.

Although both channels are very similar in sequence and structure, they differ widely in their expression patterns and functions (26–28) and it is formally possible that they are regulated by different mechanisms. KV10.1 and KV10.2 share high sequence homology and the differences between them are concentrated in the C terminus, so we next tested the effect of CTTN depletion on KV10 chimeras with exchanged C termini. Both chimeric channels expressed in Xenopus oocytes were functional, showing robust currents (Fig. 4B) with kinetics and voltage dependence very similar to wild-type currents. Current amplitudes of KV10.1 bearing the C terminus of KV10.2 were no longer sensitive to CTTN depletion (145 ± 15% of control levels, n = 23, p = 0.11), whereas those of KV10.2 with the C terminus of KV10.1 showed a prominent reduction upon PTO injection (22 ± 3% of control levels, n = 16, p < 0.0005, Fig. 4B). This supports the idea that the C terminus of KV10.1 mediates the functional interaction of KV10.1 and CTTN.

To further map the binding site of CTTN within the C terminus of KV10.1, we constructed mutated channels with various C-terminal deletions (KV10.1 Δ1–8, Fig. 4C). Deletions were chosen in those regions that differ most between KV10.1 and KV10.2. As depletion of endogenous CTTN greatly reduced the KV10.1-mediated current, we expected the deletion of the CTTN binding site to have the same effect. Indeed, we found that five of the eight deletion mutants produced reduced current amplitudes (Fig. 4C). One group of deletions, namely KV10.1 Δ4–Δ6 showed only moderate reduction of current amplitude, which was further decreased by depletion of CTTN.

FIGURE 4. Regulating effect of the CTTN expression level depends on C terminus of KV10.1. A, varying CTTN levels by depletion (PTO, left panel) or overexpression (CTTN, right panel) in Xenopus oocytes did not influence whole cell current amplitudes of KV10.2 in comparison to control levels. B, exchanging C termini between KV10.1 and KV10.2 transfers current dependence to CTTN levels. Current amplitudes of KV10.1 bearing the KV10.2 C terminus (Ba, panel a) were no longer sensitive to CTTN depletion in contrast to currents mediated by KV10.2 bearing the KV10.1 C terminus (Bb, panel b), which were significantly affected by CTTN reduction with injection of PTO. Current traces above the bar diagrams show typical responses elicited by the respective chimeras in Xenopus oocytes (see legend to Fig. 3). Scale bars: 5 μA, 200 ms. C, effect of partial deletion of the KV10.1 C terminus on current amplitudes. Partial deletions of the C terminus of KV10.1 were generated and expressed in Xenopus oocytes. Current amplitudes of deletions KV10.1 Δ3, Δ4, Δ5, Δ6, and Δ8 were significantly reduced compared with the wild type.
Cortactin Controls K_\text{v}10.1 Functional Expression

FIGURE 5. Deletion of amino acids 705–755 of K_\text{v}10.1 changes its distribution as well as its ability to bind CTTN. A, depletion of CTTN by co-injection of the PTO oligonucleotide was able to reduce current amplitudes of K_\text{v}10.1 \Delta 3 (del705–755) in voltage clamp oocyte recordings as determined by a depolarization step to +80 mV. Restoration of CTTN levels by injection of human CTTN did not rescue the effect of PTO on this mutant. B, the effect of PTO-mediated CTTN depletion on K_\text{v}10.1 \Delta 3 was weaker (reducing current amplitude by 50%) than that observed in full-length K_\text{v}10.1 (that reduced current by 80%). Current amplitude of the mutant K_\text{v}10.1 \Delta 3 after CTTN depletion was not rescued by additional expression of human CTTN, in contrast to wild-type K_\text{v}10.1. C, membrane K_\text{v}10.1-BBS was labeled using a BTX-Alexa 555 conjugate and allowed for internalization for 1 h. Washed cells were analyzed by wide field microscopy. K_\text{v}10.1-BBS as well as K_\text{v}10.1-BBS \Delta 8 (del933–962) showed prominent membranous labeling of K_\text{v}10.1-BBS, whereas nearly no membrane-bound K_\text{v}10.1-BBS \Delta 3 was observed. Scale bar, 10 \mu m. D, K_\text{v}10.1-BBS and K_\text{v}10.1-BBS \Delta 8 successfully precipitated GST-CTTN, K_\text{v}10.1-BBS \Delta 3 showed much weaker co-precipitation of the full-length CTTN. Mutants K_\text{v}10.1-BBS \Delta 3, K_\text{v}10.1-BBS \Delta 8, or wild-type K_\text{v}10.1-BBS were transiently expressed in HEK 293 cells. Lysates were labeled with BTX-biotin (Bb) to pull down K_\text{v}10.1-BBS using streptavidin-coated beads, unlabeled lysates served as negative control. Subsequent GST-CTTN was added and analyzed for co-precipitation using SDS-PAGE followed by Western blotting using a GST antibody.

(not shown). This indicates that these regions are not implicated in the K_\text{v}10.1-CTTN interaction. Deletion of amino acid residues 705–755 (K_\text{v}10.1 \Delta 3) or the extreme C terminus (K_\text{v}10.1 \Delta 8) led to a reduction of current amplitudes by more than 90% (Fig. 4C), so we concentrated on these two constructs for further analysis.

To check if depletion of CTTN has any effect on current amplitudes of the mutants we increased the amount of cRNA injected to obtain quantifiable currents, and then measured the expression at the plasma membrane in a cell culture system by adding the BBS tag to the deletion mutants. Living HEK 293 cells expressing K_\text{v}10.1-BBS, K_\text{v}10.1 \Delta 3-BBS, or K_\text{v}10.1 \Delta 8-BBS were labeled with a BTX-Alexa Fluor conjugate to visualize membrane and internalized K_\text{v}10.1 (19). A clear membrane staining as well as internalized K_\text{v}10.1 was seen independently of the presence of the extreme C terminus of K_\text{v}10.1 (Fig. 5C, K_\text{v}10.1-BBS and K_\text{v}10.1-Δ8-BBS). Despite robust expression levels of all three constructs, only K_\text{v}10.1 \Δ3-BBS showed reduced labeling; internalized K_\text{v}10.1 was stained, but there was minimal signal at the membrane (Fig. 5C). With our experimental protocol, only surface BBS can be detected. Therefore, there is probably a population of channels at the surface, but we were not able to detect it. Close-ups of the regions indicated by a white rectangle are shown in the lower panel. Peripheral staining was prominent in K_\text{v}10.1-BBS, whereas only punctate structures could be detected in K_\text{v}10.1 \Δ3-BBS. K_\text{v}10.1 \Δ8-BBS showed an intermediate profile with clear staining of cellular processes, compatible with plasma membrane taken together with abundant punctate staining.

Precipitation studies with GST-CTTN using K_\text{v}10.1-BBS \Δ3 or \Δ8, as described above, revealed, that K_\text{v}10.1 \Δ8 is able to bind GST-CTTN with an efficiency comparable with wild-type K_\text{v}10.1, whereas K_\text{v}10.1 \Δ3 showed reduced binding capability (Fig. 5D), although co-precipitation of the HP-region of CTTN...
Cortactin Controls KV10.1 Functional Expression

A

B

C

D

Pulldown membrane KV10.1-BBS

FIGURE 6. CTTN influences surface expression of KV10.1. A, non-stationary noise analysis of KV10.1-mediated current in control and CTTN-depleted Xenopus oocytes. Representative plots of the variance against the mean current with the corresponding fitting parabola for patches derived from control untreated (squares) or CTTN-depleted (circles) oocytes. The same plots are shown with independent scales in the inset. The slope of the initial segment of the function (representing single channel current) did not change upon CTTN depletion. B, depletion of CTTN reduced surface levels of KV10.1. Expression of KV10.1 in Xenopus oocytes was analyzed by Western blot (WB) using anti-KV10.1 antibodies. No difference of KV10.1 expression was found in whole oocyte lysates in response to CTTN depletion with the PTO (left panel), whereas surface levels of KV10.1 obtained from pull-down assays of membrane bound KV10.1-BBS were clearly reduced in the presence of the PTO (right panel).

was observed for KV10.1 A3 (not shown). We therefore conclude that amino acid residues 705–755 of KV10.1 contribute to the functional interaction with CTTN, but it is likely that other unidentified regions also participate in CTTN binding.

CTTN Does Not Influence the Biophysical Properties of KV10.2—Total current through an ion channel results from the combination of three parameters: the unitary current through a single channel (i), the total number of available channels (N), and the probability of a channel being open (P_o).

\[ I = iNP_o \]  
(Eq. 2)

Thus, a change in I can be caused by altering channel surface expression (varying N) or eliciting a change in single channel conductance or open probability. Each of these possibilities is conceivable in the case of CTTN and KV10.1. The cytoskeleton is known to play an important role in regulating the KV10.1-mediated current (15) and CTTN is reportedly involved in the regulation of the surface expression of some ion channels and receptors (8, 30); it can also alter the conducting properties of KCa1.1 channels by regulating its interactions with the actin cytoskeleton (6, 7). To distinguish between these possibilities, we used electrophysiological and biochemical techniques.

To determine electrophysiological which of the above parameters is altered, we used non-stationary noise analysis, a technique that allows an estimation of I, N, and P_o, from macroscopic currents based on the variance observed in the current trace. Variance is minimal both when all channels are either closed or open (see “Experimental Procedures”). We performed such analysis on excised Xenopus oocyte macropatches in the outside-out configuration. We averaged 300 depolarizing stimuli and determined point by point the variance of each trace with respect to the average trace. Fitting a parabola function (Equation 1) to the current versus the variance data, the slope of the initial, pseudolinear segment of the distribution corresponds to the single channel current (23). The result of the fitting gave similar single channel currents in the presence (884 ± 64 fA) and absence (789 ± 68 fA, p = 0.4) of CTTN (Fig. 6A). When normalized to compensate for the smaller currents in patches from PTO-treated oocytes, the variance versus current plots overlapped (Fig. 6A, inset). Under the experimental conditions, the maximal open probability reached was similar in the presence or absence of CTTN (0.58 ± 0.03 versus 0.57 ± 0.04, p = 0.4; Fig. 6C). Therefore, the parameter that is most likely influenced by CTTN knockdown is the number of available channels. These data strengthen the idea that CTTN influences the number of functional KV10.1 channels at the plasma membrane.

Surface Expression of KV10.1 Depends on the Presence of CTTN—We next analyzed the expression levels of KV10.1 at the plasma membrane with biochemical techniques. Insertion of a BBS at the extracellular part of KV10.1 allows specific labeling of membranous KV10.1-BBS in intact cells (19); cRNA encoding these modified proteins was injected into oocytes. Exposed KV10.1-BBS was labeled in the intact oocyte with BTX-biotin. Oocytes were then lysed, and labeled KV10.1-BBS was pulled down using streptavidin. In agreement with the electrophysiological data, CTTN-depleted cells showed a clear reduction in labeled KV10.1-BBS, indicating less protein at the cell surface in three independent experiments (Fig. 6B). Importantly, the total protein level of KV10.1 in whole oocyte lysates was independent of CTTN levels (Fig. 6B, right panel), arguing against the possibility of reduced surface expression due to a reduction in the protein in the cell. Taken together, these
results strongly suggest that CTTN regulates surface expression of Kv10.1 in Xenopus oocytes.

We then asked if observations made in oocytes could be translated to mammalian expression systems more suitable for studying trafficking processes. For this purpose, we first examined if alterations in CTTN expression levels induce changes in the cellular localization of Kv10.1 in mammalian cells. Cells with normal, reduced, or increased levels of CTTN were transfected with a fluorescent Kv10.1-Venus fusion protein and analyzed by confocal microscopy. Overexpression of CTTN showed enrichment of the Kv10.1 signal in focal adhesions. This distribution was lost upon CTTN knockdown. Scale bars, 20 μm.

Depletion of CTTN had a very similar effect to deletion of Kv10.1-BBS (tM, 0.29 ± 0.03, Table 1) or Kv10.2-BBS (tM, 0.31 ± 0.09). At the membrane or presumptive focal adhesions, no co-localization of these two proteins was observed (Fig. 7A), again indicating that CTTN interacts with neither Kv10.2 nor Kv10.1 ∆3-BBS in vivo.

We also labeled and tracked Kv10.1-BBS in living cells using fluorescently labeled BTX. HEK 293 cells stably expressing Kv10.1-BBS were transfected either with control (scrRNA) or siRNA directed against CTTN. Reduced CTTN levels to about 50% (as revealed by Western blot analysis, Fig. 8A) lowered surface staining by BTX (Fig. 8B). To quantify this effect, we performed ELISA on BTX-coated plates (by immobilization of BTX-biotin on streptavidin-coated plates). Labeled cell extracts were incubated on these plates and Kv10.1 was detected using a C-terminal antibody. Different labeling protocols enabled us to distinguish between different fractions of Kv10.1-BBS. Incubating with BTX after cell lysis labels total Kv10.1 and not just the exposed fraction. Quantification showed that changing the CTTN expression levels did not have any effect on the total Kv10.1 content (siRNA mediated knockdown: 105 ± 5% of the control, overexpression: 97 ± 5% of control; Fig. 8C), indicating that, like in oocytes, CTTN does not affect the expression levels of Kv10.1.

Selective labeling and detection of membrane-bound Kv10.1-BBS can be achieved by incubating living cells with the conjugate at room temperature for a short time to avoid internalization (19). Comparable with the findings in oocytes, overexpression of CTTN did not increase the membrane fraction of Kv10.1-BBS (112 ± 21% of control), whereas siRNA-mediated knockdown of CTTN (to ~50%, as determined for every individual experiment) significantly reduced surface levels of Kv10.1-BBS (57 ± 5% of the control, Fig. 8C). Taken together, these results point to a CTTN-mediated regulation of Kv10.1 surface expression.

CTTN Stabilizes Kv10.1 at the Cell Membrane—As the amount of total Kv10.1 does not change in response to alterations in CTTN expression, two mechanisms to vary Kv10.1 membrane expression are conceivable. If CTTN is involved in trafficking processes of Kv10.1 toward the membrane, less Kv10.1 may reach the membrane when CTTN is missing. Alternatively, in the absence of CTTN, Kv10.1 that had reached the membrane would remain there for a shorter time, thus reducing the steady-state level.

If a trafficking process is involved, one would expect that depletion of CTTN prior to Kv10.1 expression would reduce the membrane expression of Kv10.1. To test this, sequential injection of the PTO and Kv10.1 cRNA was therefore performed in Xenopus oocytes. When Kv10.1 cRNA was injected 24 h before CTTN levels were lowered by PTO, Kv10.1-mediated currents were significantly reduced to 51 ± 11% of control.
levels (Fig. 8D). In contrast, when PTO injection was performed before KV10.1 injection, there was no reduction of the KV10.1 current with respect to the control (92 ± 9% (Fig. 8D)). This observation argues against involvement of CTTN in trafficking processes, but instead indicates a role in stabilizing KV10.1 at the plasma membrane.

We therefore tested internalization processes in mammalian cells using ELISA experiments similar to those described previously. Living HEK293 cells stably expressing KV10.1-BBS were incubated with a BTX-biotin conjugate for 1 h at physiological temperature to allow internalization. Subsequently, membrane labeling was removed by an acid wash. Overexpression of CTTN reduced internalized KV10.1-BBS in the given time, whereas depletion of CTTN did not have an effect. D, reduction of KV10.1 current amplitude in Xenopus oocytes was only observed when CTTN depletion was achieved when PTO was injected before or simultaneously with KV10.1, but not if it was injected 24 h later. E, growth of HeLa cells was stimulated by CTTN transfection (circles) as compared with transfection with empty vector (triangles). The inset shows that knockdown of KV10.1 does not impair proliferation of CTTN-overexpressing cells.

DISCUSSION

In this study we report that CTTN is an interaction partner of the voltage-gated potassium channel KV10.1. Our results indicate that this interaction is direct as precipitation with purified proteins did not require the addition of any bridging factor. Different pulldown assay formats allowed us to identify the proline-rich region of CTTN as responsible for binding to KV10.1. This region appears to be a major substrate for post-translational modifications in CTTN, bearing several well known phosphorylation sites. We have not examined possible post-translational regulation of the CTTN interaction with KV10.1 and this does represent an area for further work.

We also observed co-localization of CTTN with KV10.1 in vivo and in Xenopus oocytes we identified CTTN expression as a requirement for the KV10.1 current, as complete depletion of...
CTTN abolished current responses. The abundance of CTTN, however, does not appear to be a limiting factor for Kv10.1 functional expression, as overexpression of CTTN could not further enhance current amplitudes in oocytes, but it did accelerate proliferation of HeLa cells, a phenomenon that has been related to Kv10.1 expression (31). This effect is specific for Kv10.1, because its closest relative, Kv10.2, did not co-localize with CTTN in vivo, nor is Kv10.2-mediated current affected by CTTN knockdown.

The known role of CTTN in actin cytoskeleton rearrangements (reviewed in Ref. 1) is unlikely to be responsible for its effects on Kv10.1 reported here; the effect was highly specific and did not apply to Kv10.2-mediated current. In addition, interference with actin cytoskeleton rearrangement through Arp2/3 inhibition did not mimic the effect of CTTN knockdown.

CTTN plays a minor role in events of Kv10.1 internalization (32) because we could detect internalized BTX in cells expressing this mutant. Additionally, the N terminus of the protein has also been implicated in tetramerization (34) in Kv11 channels. Altogether, these observations point to a scenario where the multimeric assembly occurs co-translationally with multiple interaction domains (N terminus, S6, proximal domain, and finally TCC “locking” and stabilizing the mature structure). The mature protein is then targeted to the plasma membrane and CTTN would stabilize it there. Nevertheless, residues 705–755 are probably not the only determinants of interaction with CTTN, because we still observed binding of the HP region to the deletion mutant as well as a residual effect on current amplitudes by CTTN depletion in Xenopus oocytes, pointing toward additional requirements for CTTN interaction.

CTTN can direct Kv10.1 to focal adhesions. These structures play a role in cancer via signaling cascades involving integrins as well as the FAK, a non-receptor tyrosine kinase mainly restricted to focal adhesions. These proteins seem to be key regulators in cancer progression (reviewed in Ref. 35). Again, a close relative of Kv10.1, HERG, (Kv11.1) is directly linked to β1 integrin signaling pathways, where it modulates downstream signaling elicited by integrin activation (36–38). Kv10.1, in addition to its presence at focal adhesions shown here seems to adopt a function in adhesion processes: its expression clearly influences adhesion of cells and the extracellular matrix is also able to modulate the Kv10.1-mediated current, although a direct interaction with β1 integrin has not been shown (39). The strong association of Kv10.1 with the actin cytoskeleton (39) might explain this behavior, and CTTN could serve to modulate interaction.

Other ion channels such as Kv1.2 and the large conductance calcium- and voltage-activated potassium channel KCa1.1 have been reported to bind to and to be influenced by CTTN via effects on affecting membrane expression or changes in open probability, respectively (4–8). In the latter, CTTN was suggested to bridge KCa1.1 channels to actin filaments, a process dependent on the phosphorylation state of CTTN and linkage of KCa1.1 channels to intracellular signaling (6, 7). Kv10.1 has been shown to be associated with actin fibers in CHO cells (39) and the Kv10.1-mediated current is influenced by interactions with the cytoskeleton as disruption of the actin cytoskeleton by cytochalasin B dramatically increases Kv10.1 current density (15).

In our study, examination of the biophysical properties of Kv10.1 revealed that CTTN expression levels do not influence the single channel conductance or the open probability of this channel. Together with the fact that CTTN depletion clearly reduced membrane-located Kv10.1 in Xenopus oocytes as well as in HEK 293 cells stably expressing Kv10.1-BBS, we conclude that CTTN has a regulatory effect on channel synthesis, maintenance, or transport. As CTTN depletion resulted in no detectable change in the amount of total whole cell Kv10.1, it is unlikely that CTTN affects channel synthesis or degradation. Sequential injection in Xenopus oocytes of Kv10.1 and the PTO affecting CTTN suggests that Kv10.1 transport toward the membrane is not affected by CTTN depletion. In line with this, the same amount of Kv10.1-BBS seems to reach the membrane in a given time span in ELISA experiments on CTTN-depleted HEK 293 cells. Therefore, we propose that CTTN is involved in Kv10.1 endocytotic processes or in their regulation.

Consistent with this hypothesis, CTTN depletion is known to reduce endocytosis over clathrin-coated pits (35, 40, 41). However, in our system, CTTN depletion affects Kv10.1 surface expression, leading to clearly reduced membrane-resident protein. A similar effect is reported for Kv1.2 and interpreted to be the result of stabilization of the channel at the membrane as well as favoring its endocytosis in a phosphorylation-dependent manner (8). As the amount of internalized Kv10.1 is not altered by depletion of CTTN, but overexpression of CTTN does reduce the amount of internalized Kv10.1, we believe that CTTN plays a minor role in events of Kv10.1 internalization under basal conditions. Instead, we postulate a model whereby CTTN stabilizes Kv10.1 at the membrane; in the absence of CTTN, this would lead to a reduced membrane-residency time of Kv10.1 via faster internalization. Mechanistically, CTTN may connect Kv10.1 to the cytoskeleton by directing and stabilizing it at the desired locations and its ability to bind and induce polymerization of actin (reviewed by Ref. 4).
Additionally, CTTN has been linked to angiotensin II-induced tissue repair in smooth muscle cells, influencing proliferation and migration in an insulin-like growth factor-1 receptor and Src dependent way (42). Within this process, CTTN is phosphorylated and subsequently translocated to the membrane, especially to focal adhesions. The Kv10.1-mediated current has been reported to be up-regulated in MCF-7 cells following stimulation with insulin-like growth factor-1 (13), and the pro-proliferative effects of this growth factor are dependent on Kv10.1. Kv10.1 overexpression alone has also been reported to increase proliferation rates (9); this increase requires functional ion channels at the plasma membrane because blockade by an extracellular antibody reduced this effect (43), but does not depend strictly on potassium permeation, because a point mutant unable to permeate K⁺ recapitulates many of the effects of wild-type Kv10.1, although less efficiently (44). In good agreement with these observations, we found that overexpression of CTTN in HeLa cells increased proliferation, and rendered the cells no longer sensitive to Kv10.1 knockdown.

Overall, our observations would be compatible with a model where signaling pathways such as insulin-like growth factor-1R/Akt or Src induce CTTN-mediated alteration of Kv10.1 expression at the cell surface. In this way CTTN activity could be translated into cell cycle progression. Further work will be necessary to determine whether this is the case.

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