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# Spatiotemporal Regulation of Chloride Intracellular Channel Protein CLIC4 by RhoA

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**Chloride intracellular channel (CLIC) 4 is a soluble protein structurally related to omega-type glutathione-S-transferases (GSTs) and implicated in various biological processes, ranging from chloride channel formation to vascular tubulogenesis. However, its function(s) and regulation remain unclear. Here, we show that cytosolic CLIC4 undergoes rapid but transient translocation to discrete domains at the plasma membrane upon stimulation of $G_{13}$-coupled, RhoA-activating receptors, such as those for lysophosphatidic acid, thrombin, and sphingosine-1-phosphate. CLIC4 recruitment is strictly dependent on $G\alpha_{13}$-mediated RhoA activation and F-actin integrity, but not on Rho kinase activity; it is constitutively induced upon enforced RhoA-GTP accumulation. Membrane-targeted CLIC4 does not seem to enter the plasma membrane or modulate transmembrane chloride currents. Mutational analysis reveals that CLIC4 translocation depends on at least six conserved residues, including reactive Cys35, whose equivalents are critical for the enzymatic function of GSTs. We conclude that CLIC4 is regulated by RhoA to be targeted to the plasma membrane, where it may function not as an inducible chloride channel but rather by displaying Cys-dependent transferase activity toward a yet unknown substrate.**

## INTRODUCTION

Chloride intracellular channels (CLICs) are small proteins that are structurally homologous to the omega class of glutathione transferases (GSTs) (Board *et al*., 2000; Dulhunty *et al*., 2001; Harrop *et al*., 2001; Littler *et al*., 2005) but that seem to have distinct cellular functions. Although CLIC proteins lack a recognizable signal sequence, they can induce chloride currents in artificial membrane systems under nonreducing conditions. This has led to the hypothesis that soluble CLICs can adopt an integral membrane conformation to form chloride channels under certain conditions (Cromer *et al*., 2002; Ashley, 2003; Littler *et al*., 2004, 2005). However, the channel hypothesis remains a matter of debate, and it seems likely that CLIC proteins have alternate cellular functions that are distinct from their proposed roles as chloride channels (Berryman and Bretscher, 2000; Ashley, 2003).

The mammalian CLIC family consists of six genes, named CLIC1–6. The CLIC4 protein (253 amino acids) is the best-studied family member. It is ubiquitously expressed and has been reported to localize to various subcellular compartments, including organelles, the cortical actin cytoskeleton, the plasma membrane, vesicles, and centrosomes, depending on the cell type examined (Chuang *et al*., 1999; Edwards, 1999; Fernandez-Salas *et al*., 2002; Proutski *et al*., 2002; Berryman and Goldenring, 2003; Suh *et al*., 2004). In addition to inducing anion channel activity, CLIC4 has been implicated in such diverse biological processes as keratinocyte differentiation, apoptosis, receptor trafficking, endothelial vacuole formation, and tubulogenesis (Bohman *et al*., 2005; Suh *et al*., 2007; Maeda *et al*., 2008; Tung *et al*., 2009; Ulmasov *et al*., 2009). In *Caenorhabditis elegans*, the CLIC-like EXC-4 protein is essential for the formation and maintenance of the excretory tube, but the underlying mechanism is not understood (Berry *et al*., 2003; Berry and Hobert, 2006). CLIC4 interacts with brain dynamin-I in a complex with actin and tubulin, suggesting a possible role for CLIC4 in membrane trafficking and/or cytoskeletal organization (Suginta *et al*., 2001). At the molecular level, residue Cys35 is of particular interest because it is highly reactive (Ashley, 2003; Littler *et al*., 2005; Weerapana *et al*., 2008) and conserved in the omega-GSTs, where it functions as an active-site residue (Board *et al*., 2000; Whitbread *et al*., 2005).

Here, we set out to determine whether CLIC4 may function in one or more receptor-linked signaling pathways, particularly those involving $Cl^-$ channel activation and cytoskeletal remodeling. By using a variety of experimental approaches, including time-lapse microscopy, patch-clamp electrophysiology, and site-directed mutagenesis, we show that CLIC4 is regulated by the $G_{13}$-linked RhoA pathway and is targeted specifically to RhoA-activating receptor complexes at the plasma membrane. Our results define CLIC4 as a novel player in RhoA signaling and suggest that it functions not as an inducible plasma membrane $Cl^-$ channel but rather by displaying Cys-dependent catalytic activity toward a yet-to-be discovered substrate.

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Abbreviations used: CLIC, chloride intracellular channel; GPCR, G protein-coupled receptor; GST, glutathione transferase; LPA, lysophosphatidic acid.

## MATERIALS AND METHODS

### Reagents

Pertussis toxin (PTX) was from List Biological Laboratories (Campbell, CA). Thrombin receptor-activating peptide (TRP; sequence SFLRRN) was synthesized in-house. C3 exo-enzyme was kindly provided by Dr. Shuh Narumiya (Kyoto University, Kyoto, Japan). CLIC4 polyclonal antibody is directed against the N terminus of CLIC4 (Berryman and Bretscher, 2000). Hemagglutinin (HA) rat monoclonal (3F10) was from Roche Diagnostics (Indianapolis, IN); green fluorescent protein (GFP) polyclonal antibody was generated in-house; horseradish peroxidase-conjugated secondary antibodies were from Dako North America (Carpinteria, CA). Other reagents were from Sigma-Aldrich (St. Louis, MO).

### Cell Culture

N1E-115, human embryonic kidney (HEK) 293, Rat-1, HeLa, and A431 cells were grown in serum-containing DMEM. Cells were seeded and cultured on glass coverslips. Constructs were transiently transfected using FuGENE 6 transfection reagent (Roche Diagnostics). Experiments were performed in a culture chamber mounted on an inverted microscope in bicarbonate-buffered saline (140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM glucose, 23 mM $NaHCO_3$, and 10 mM HEPES pH 7.2) and kept under 5% $CO_2$ at 37°C.

### DNA Constructs

For generation of GFP-CLIC4, CLIC4 cDNA was isolated from human placenta (clone 11-1a) by using a forward primer with a BamHI adaptor immediately preceding the endogenous start codon (CGCG/GATCC ATG GCG TTG TCG ATG CCG C) and a reverse primer with a HindIII adaptor immediately following the endogenous stop codon (GCA/AGCTT TTA CTT GGT GAG TCT TTT GGC). Polymerase chain reaction (PCR) product was subcloned into PCR2.1 Topo vector and from this a Bam-HI insert was ligated into peGFP-C1 vector and verified for proper orientation by sequencing. Cyan fluorescent protein (CFP)-, yellow fluorescent protein (YFP)-, and HA-CLIC4 were made by substituting green fluorescent protein (GFP) of GFP-CLIC4 with fluorophores from peCFP-C1, peYFP-C1 (NheI/KpnI), or oligonucleotides containing coding sequence for HA flanked by restriction sites NheI/KpnI. For fluorophore-tagging at the C terminus, CLIC4 insert was isolated from template GFP-CLIC4 by PCR (forward primer AGCTGATATCTGATG-GCGTTGTCGAT; reverse primer AAAAGACTCACCAAGGCTAGCAGCT) and ligated (EcoRV/NheI) into pcDNA3.1 vectors containing YFP or mCherry C-terminal to a multiple cloning site. Point mutations in YFP-CLIC4 were generated using Phusion site-directed mutagenesis kit (Finnzymes, Espoo, Finland) and verified by sequencing analysis. Generation of GFP-CLIC1 and CLIC1-YFP was identical to the approach followed for CLIC4 constructs. DsRed-NHERF2 was a kind gift from Dr. T. Gadella (University of Amsterdam, Amsterdam, The Netherlands), pSuper-RNAi-RhoA was obtained from Dr. J. Collard (The Netherlands Cancer Institute, Amsterdam, The Netherlands).

### RNA Interference

Small-hairpin RNA constructs were generated by insertion (BglII/HindIII) of oligonucleotides into the pSuper vector and verified by sequencing analysis. Target sequences are listed in Supplemental Table S1. Transfections were done using FuGENE and a mix of pSuper vectors (listed in Supplemental Table 1).

### Time-Lapse Microscopy and Image Analysis

Coverslips with cells expressing various constructs were mounted in a culture chamber and imaged using an inverted TCS-SP5 confocal microscope equipped with 63× immersion oil lens (numerical aperture 1.4; Leica, Mannheim, Germany). Imaging conditions were as follows: CFP, excitation at 442 nm and emission at 465–500 nm; GFP, excitation at 488 nm and emission at 510–560 nm; YFP, excitation at 514 nm and emission at 522–570 nm; and mCherry, excitation at 561 nm and emission at 580–630 nm. For translocation studies, confocal images were taken at 5- or 10-s intervals. To quantitatively express the translocation of GFP- or YFP-CLIC4, the ratio of plasma membrane (PM)-to-cytosolic fluorescence was calculated after acquisition by automated assignment of regions of interest by using Qwin software (Leica) (van der Wal *et al*., 2001).

### Immunofluorescence

Cells were fixed by electron microscopy (EM)-grade paraformaldehyde (PFA) or (for endogenous CLIC4) methanol, permeabilized using 0.1% Triton X-100, blocked in 2% bovine serum albumin, and incubated with primary antibody and subsequently with Alexa-conjugated secondary antibodies. Mounted slides were examined on a TCS-SP5 confocal microscope (63×; Leica). Experiments to detect externalized HA-epitope of overexpressed HA-CLIC4 were performed under strictly nonpermeabilizing conditions, i.e., without Triton X-100 treatment by using PFA as a fixative. Because even optimized conditions cannot exclude some plasma membrane leakage, we verified plasma membrane integrity for every studied cell by costaining of cotransfected H2B-CFP. This internal control showed immunostained H2B-CFP in ~2% of the cells, underlining the risk of false positive results.

### Membrane Potential Measurements

N1E-115 cells were loaded with voltage-sensitive dye (FLIPR membrane potential assay kit, catalog no. R8128; Molecular Devices, Sunnyvale, CA) for 5–10 min and then mounted on an inverted microscope (40×; Carl Zeiss, Thornwood, NY). Excitation was at 515 nm from a monochromator Hg-lamp. Fluorescence (long-pass filtered >540 nm) was detected with a photon multiplier tube. Excitation intensity was adapted to yield a standardized baseline output signal. Diaphragms were used to collect emission selectively from transfected cells (discriminated by H2B-CFP transfection marker at 425 nm excitation).

### Patch-Clamp Measurements

Electrophysiological recordings were made essentially as described previously (Postma *et al*., 1996a). Current recordings were digitized at 100 kHz or 10 Hz (steady-state whole-cell currents). Borosilicate glass pipettes were fire polished to 5–7 MΩ. After establishment of the giga-ohm seal, the patched membrane was ruptured by gentle suction to obtain whole-cell configuration. Solutions were as follows: patch pipette included 120 mM K-glutamate, 30 mM KCl, 1 mM $MgCl_2$, 0.2 mM $CaCl_2$, 1 mM EGTA, 10 mM HEPES, pH 7.2, and 1 mM ATP; and bath solution included 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, pH 7.3, and 10 mM glucose.

### SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting

Cells were harvested in Laemmli sample buffer, boiled for 10 min, and subjected to immunoblot analysis according to standard procedures. Filters were blocked in Tris-buffered saline/Tween 20 and 5% milk, incubated with primary and secondary antibodies, and visualized by enhanced chemoluminescence (GE Healthcare, Chalfont St. Giles, Buckinghamshire, United Kingdom).

## RESULTS

To explore whether CLIC4 may function in cellular signaling and/or chloride channel formation, we used mouse N1E-115 neuroblastoma cells as a model because receptor signaling, ion channel activation, and cytoskeletal remodeling have been well characterized in these cells (Jalink *et al*., 1994; Postma *et al*., 1996a; Hirose *et al*., 1998; Kranenburg *et al*., 1999; Postma *et al*., 2001). Most, if not all, mammalian tissues and cell types endogenously express CLIC4, along with other CLIC family members. PCR analysis revealed that N1E-115 cells express CLIC1, CLIC4, and CLIC6 (data not shown).

### Translocation of CLIC4 toward the Plasma Membrane: Regulation by the $G_{13}$-linked RhoA Pathway

To characterize the spatiotemporal regulation of CLIC4 by time-lapse microscopy, we generated constructs of CLIC4 fused to various fluorophores. When expressed in N1E-115 cells, GFP-tagged CLIC4 was distributed homogenously through the cytosol (Figure 1A). On addition of serum (5%) or 1 $\mu$M lysophosphatidic acid (LPA), a major serum constituent acting on specific G protein-coupled receptors (GPCRs) (Moolenaar *et al*., 2004), we observed a rapid translocation of GFP-CLIC4 toward discrete domains at the plasma membrane, concomitant with depletion of CLIC4 from the cytosol (Figure 1A and Supplemental Video 1). Interestingly, GFP-CLIC4 accumulation at the plasma membrane was transient: it was maximal by ~1 min and had disappeared at 5–10 min after LPA addition (Figure 1B and Supplemental Video 1). A similar translocation was observed with C-terminally tagged CLIC4-YFP (data not shown). Importantly, this subcellular relocalization was also observed for endogenous CLIC4 (Figure 1C), indicating that the GFP-tag has little or no effect on CLIC4 translocation. Closer inspection of the confocal images revealed that CLIC4 depletion from the cytosol occurred

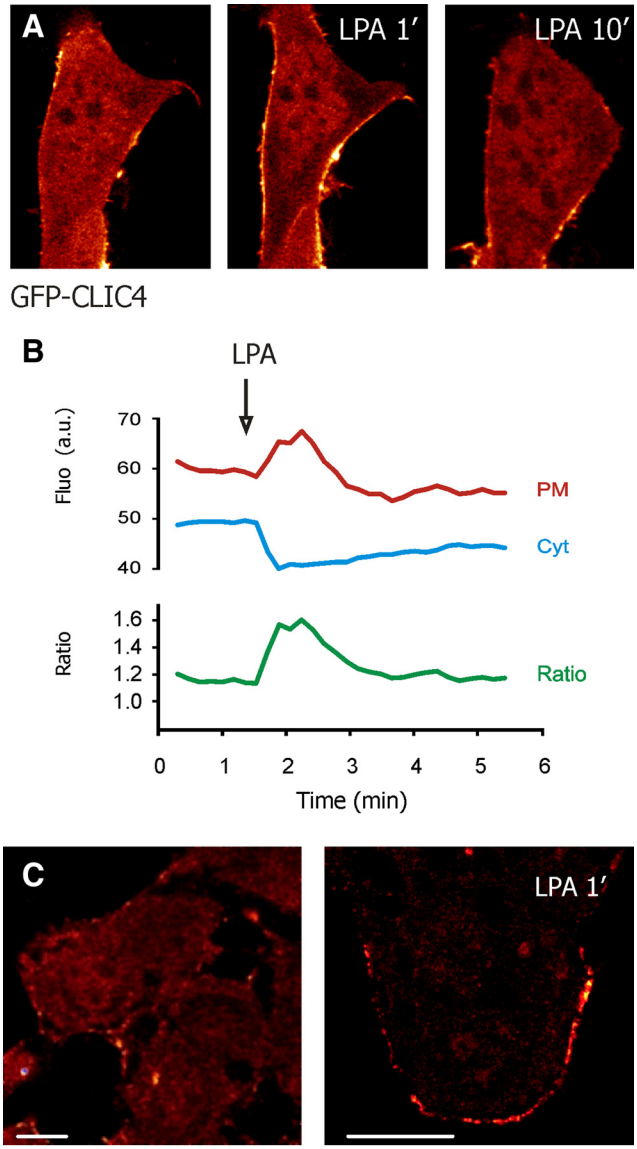



**Figure 1.** Agonist-induced translocation of CLIC4 toward the plasma membrane. (A) LPA-induced translocation of GFP-CLIC4 in N1E-115 cells visualized by time-lapse microscopy. In resting cells, GFP-CLIC4 resides mainly in the cytosol with some patch-like accumulation at the cell periphery. LPA (1 $\mu$M) induces a rapid but transient recruitment of GFP-CLIC4 toward the plasma membrane. Similar responses were observed with fetal calf serum (5%), S1P, and TRP (see Table 1; also see Supplemental Video 1). Bar, 10 $\mu$m. (B) GFP-CLIC4 translocation in response to 1 $\mu$M LPA measured semiquantitatively by monitoring the accumulation of GFP fluorescence at the PM (red trace) and the concomitant depletion of fluorescence from the cytosol (Cyt; blue trace). Net translocation is expressed as the ratio PM/Cyt (green trace). (C) Distribution of endogenous CLIC4 in resting versus LPA-stimulated N1E-115 cells. Plasma membrane accumulation was strongly increased after 1 min of 1 $\mu$M LPA addition. Bars, 10 $\mu$m.

in a homogeneous manner, suggesting that cytosolic CLIC4 is freely diffusible (Figure 1A and Supplemental Video 1). Indeed, fluorescence recovery after photobleaching experiments showed that cytosolic GFP-CLIC4 is as mobile as free GFP (data not shown).

**Table 1.** CLIC4 translocation, $Cl^-$-mediated membrane depolarization, and RhoA-mediated cytoskeletal contraction under various conditions[a]

| Agonist/treatment | CLIC4 translocation | $Cl^-$ channel activation (depolarization) | Cytoskeletal contraction (RhoA mediated) |
|---|---|---|---|
| Serum (5%) | ++ | ++ | ++ |
| LPA, S1P, TRP[b] | ++ | ++ | ++ |
| Bradykinin,[c] isoprotenerol | − | − | − |
| LPA | | | |
| + Pertussis toxin (200 ng/ml) | ++ | ++ | ++ |
| + $G\alpha_{13}$ or RhoA knockdown | − | − | − |
| + C3 transferase (30 $\mu$g/ml) | − | − | − |
| + Y-27632 (100 $\mu$M) | ++ | ++ | − |
| + Latrunculin A (1 $\mu$M) | − | ++ | − |
| + CLIC4 knockdown | | ++ | ++ |

[a] ++, positive response; −, no response.

[b] Ligand concentrations: LPA, S1P, bradykinin, isoprotenerol, 1 $\mu$M; TRP, 50 $\mu$M.

[c] See Supplemental Figure S1.

In search of the signaling events underlying CLIC4 recruitment, we found that LPA-induced translocation is insensitive to PTX, ruling out the involvement of $G_i$-linked effector pathways. Translocation was only observed with GPCR agonists that activate the $G_{12/13}$-linked RhoA pathway in N1E-115 cells, notably, LPA, thrombin (or TRP), and sphingosine 1-phosphate (S1P) (Jalink *et al*., 1994; Postma *et al*., 1996b; Kranenburg *et al*., 1999), as summarized in Table 1. $G_q$- and $G_s$-linked receptor agonists (bradykinin and isoprotenerol, respectively) and $Ca^{2+}$ ionophore (ionomycin) all failed to trigger plasma membrane recruitment of CLIC4 (Table 1). As illustrated in Supplemental Figure S1, the $G_q$-coupled receptor agonist bradykinin evokes robust hydrolysis of phosphatidylinositol (4,5)-bisphosphate ($PIP_2$) but fails to activate RhoA and to trigger CLIC4 translocation (also see Postma *et al*., 2001). Furthermore, we note that S1P triggers RhoA activation and CLIC4 translocation (Table 1) without stimulating $PIP_2$ hydrolysis in N1E-115 cells (Postma *et al*., 2001; data not shown). Collectively, these findings rule out the involvement of $G_q$, $G_i$, $G_s$, and classic second messengers ($PIP_2$, $Ca^{2+}$, and cAMP) in regulating CLIC4 translocation.

LPA receptors couple to $G\alpha_{13}$ rather than $G\alpha_{12}$ in N1E-115 cells (Postma *et al*., 2001). As a direct test for involvement of the $G_{13}$-RhoA pathway we knocked down either $G\alpha_{13}$ or RhoA in N1E-115 cells. This abolished LPA-induced CLIC4 translocation, as did pretreatment of the cells with the Rho-inactivating C3 transferase. That RNAi and C3 treatment were effectively inhibiting RhoA signaling was evidenced by loss of agonist-induced cell rounding (Jalink *et al*., 1994) (Figure 2A; data not shown). In the converse approach, we enforced GDP/GTP exchange on RhoA by using the catalytic DH/PH domain of the Rho-specific exchange factor p190RhoGEF, which leads to robust RhoA-GTP accumulation (van Horck *et al*., 2001). As shown in Figure 2B, expression of the isolated DH/PH domain resulted in persistent

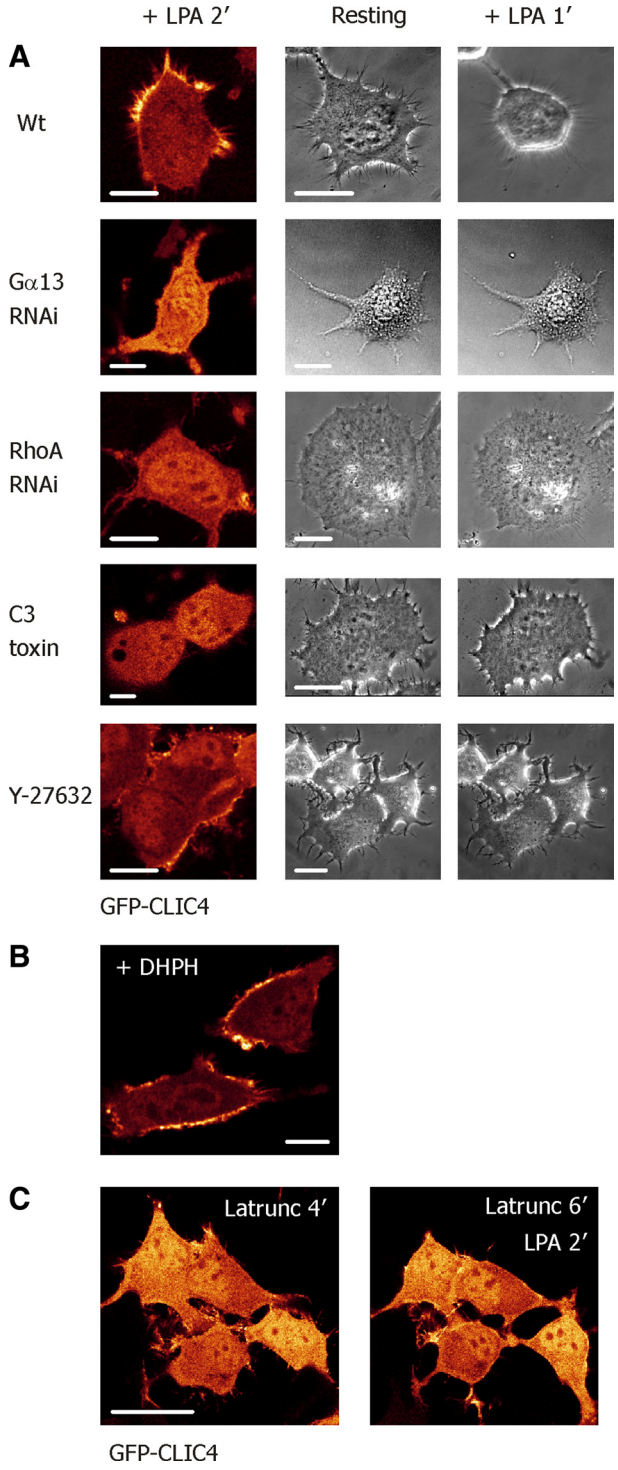



**Figure 2.** Spatiotemporal regulation of CLIC4 by RhoA. (A) Visualization of GFP-CLIC4 translocation and cell morphology under various conditions affecting Ga13-RhoA signaling in N1E-115 cells. RNA interference-encoding vectors were transfected for 48 h together with GFP-CLIC4 (transfection marker mRFP-H2B was used in the cell rounding controls; not shown in the images). C3 transferase (30 $\mu$g/ml) was incubated for 16 h and the ROCK inhibitor Y-27632 (30 $\mu$M) for 30 min. Bars, 10 $\mu$m. (B) Introduction of the catalytic DH/PH domain of p190RhoGEF, which robustly stimulates GTP loading on RhoA (van Horck *et al.*, 2001), leads to constitutive recruitment of GFP-CLIC4 at the plasma membrane. Bar, 10 $\mu$m. (C) Latrunculin A (1 $\mu$M; 4-min pretreatment) abolishes LPA-induced translocation of GFP-CLIC4. Bar, 20 $\mu$m.

accumulation of CLIC4 at the plasma membrane. From these results we conclude that CLIC4 translocation is mediated by active RhoA-GTP. Consistent with this, the transient kinetics of CLIC4 translocation (Figure 1B) are very similar to those of RhoA-GTP accumulation in LPA-treated N1E-115 cells (Kranenburg *et al.*, 1999; Couvillon and Exton, 2006).

CLIC4 translocation was also observed in Rat-1 fibroblasts, HEK293 cells, HeLa and A431 carcinoma cells stimulated with either LPA or TRP. It thus seems that CLIC4 recruitment to the plasma membrane is a common cellular response to stimulation of RhoA-activating GPCRs.

Rho GTPases regulate the cytoskeleton through their downstream effectors (Etienne-Manneville and Hall, 2002; Ridley, 2006). RhoA stimulates actomyosin-based contractility and stress fiber formation via its major effector Rho kinase (ROCK) (Hirose *et al.*, 1998). However, the ROCK inhibitor Y-27632 (10–100 $\mu$M), although fully inhibiting cytoskeletal contraction, did not affect CLIC4 translocation upon receptor stimulation (Figure 2A and Table 1). This rules out a role for ROCK in recruiting CLIC4. Conversely, knockdown of CLIC4 (blot in Figure 3C) did not detectably affect LPA-induced cell rounding (n = 20), strongly suggesting that CLIC4 is dispensable for ROCK-mediated cytoskeletal remodeling. However, agonist-induced CLIC4 translocation was completely inhibited by latrunculin A (1 $\mu$M; 4-min pretreatment), a toxin that binds monomeric actin and thereby prevents F-actin polymerization (Morton *et al.*, 2000) (Figure 2C and Table 1). It thus seems that plasma membrane recruitment of CLIC4 requires F-actin integrity but not actomyosin-based contractility.

### *CLIC4 Recruitment Can Be Dissociated from Plasma Membrane Chloride Channel Activation*

Previous studies have suggested that soluble CLICs may auto-insert into and pass through membranes to form $Cl^-$ channels, with the N terminus projecting outward and the C terminus inward (Tonini *et al.*, 2000; Proutski *et al.*, 2002; Ashley, 2003). To examine whether the N-terminal HA-tag of membrane-targeted CLIC4 becomes externalized, permeabilized and nonpermeabilized HEK293 cells overexpressing HA-CLIC4 were stained with anti-HA antibody. Truly nonpermeabilized cells did not show any specific HA-CLIC4 staining, whereas the externalized HA-tag of a classic transmembrane protein (the $\alpha$1 adrenergic receptor) was readily detected (Supplemental Figure S2). Thus, CLIC4 accumulation at the plasma membrane does not lead to externalization of the N terminus.

Even if CLIC4 does not span the plasma membrane, it remains possible that membrane-targeted CLIC4 modulates $Cl^-$ channel activity. In this respect, it is of note that the kinetics of CLIC4 recruitment are similar to those of the $G\alpha_{13}$-mediated $Cl^-$ current in N1E-115 cells (Postma *et al.*, 1996a; Postma *et al.*, 2001). This current manifests itself as a transient membrane depolarization (from approximately $-60$ to $-15$ mV), which can be monitored by a voltage-sensitive dye (Figure 3A) (Postma *et al.*, 1996a). We found that, in common with CLIC4 recruitment, LPA-induced membrane depolarization was abolished after knockdown of $G\alpha_{13}$ and RhoA as well as by C3 treatment, but not by Y-27632 (Figure 3A). Thus, CLIC4 recruitment and $Cl^-$ channel induction show overlapping features. However, the agonist-induced membrane depolarization was insensitive to latrunculin A at doses that blocked CLIC4 recruitment (Figure 3B). Thus, agonist-induced $Cl^-$ channel activation can be dissociated from CLIC4 recruitment. Next, we compared $Cl^-$ currents in normal and CLIC4-depleted N1E-115 cells by using the patch-clamp technique. However, neither the

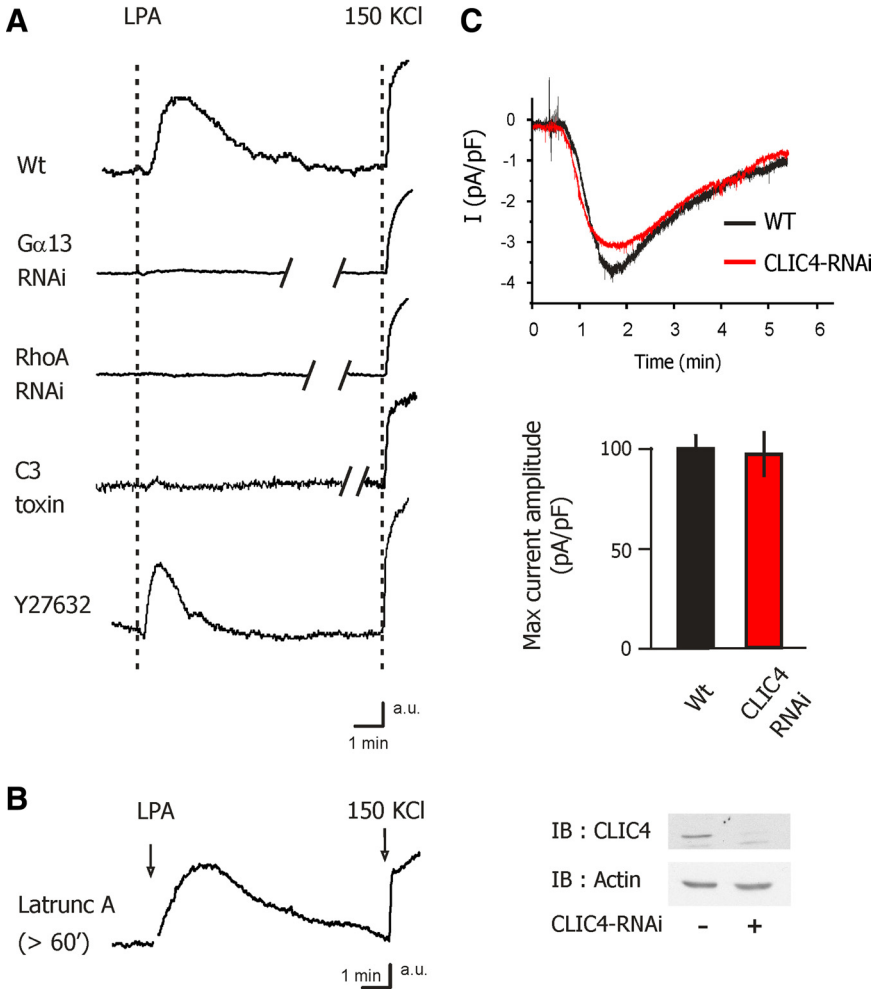



**Figure 3.** LPA-induced membrane depolarization and inward currents. (A) LPA-stimulated N1E-115 cells undergo transient $Cl^-$-mediated membrane depolarization as measured by a voltage-sensitive dye (see *Materials and Methods*). Indicated RNA interference or drug treatments correspond to those shown in Figure 2A ($n > 100$) (transfection marker CFP-H2B). End-of-experiment calibration was done by using 150 mM KCl, which eliminates the transmembrane potential. (B) Latrunculin A (1 $\mu$M; up to 1 h pretreatment) does not affect LPA-induced membrane depolarization, whereas it abolishes CLIC4 translocation (see Figure 2B). Recording is representative of eight experiments. (C) Representative patch-clamp recordings of inward $Cl^-$ currents in LPA-stimulated N1E-115 cells. Membrane potential was clamped at −60 mV (for details, see Postma *et al*., 1996a). Black trace, untreated cells. Red trace, CLIC4-depleted cells (three RNA interference targeting constructs). The bar diagram shows peak amplitudes (mean ± SEM). Transmembrane currents were corrected for membrane conductance, which is a measure for total cell surface area (pA/pF). Bottom, Western blots showing CLIC4 levels in control and knockdown N1E-115 cells. Actin was used as loading control.

kinetics nor the amplitude of the LPA-induced inward $Cl^-$ current was affected by depleting CLIC4 (Figure 3C). These results strongly suggest that CLIC4 does not modulate $Cl^-$ currents in LPA-stimulated cells.

### *CLIC4 Translocates toward Activated Receptor Complexes*

When comparing CLIC4 translocation induced by LPA to that induced by TRP, we noticed striking differences in the patterns of CLIC4 accumulation. LPA stimulation generally resulted in an asymmetric and highly polarized distribution of CLIC4 along the plasma membrane, whereas TRP-stimulated cells showed CLIC4 patches at multiple regions along the cell periphery. This is exemplified by experiments in which the same cells were first stimulated with TRP and after 10 min with LPA. This resulted in markedly different localizations of GFP-CLIC4 (Figure 4A and Supplemental Video 2). Such differential distribution was also observed for endogenous CLIC4 in cells stimulated with LPA or TRP (Figure 4A). We hypothesized that this differential CLIC4 localization reflects the differences in membrane localization of the respective GPCRs.

We tested this hypothesis by three different approaches. First, we took advantage of the fact that N1E-115 cells endogenously express $LPA_2$ receptors, which are known to form a complex with the scaffold protein NHERF2 (Oh *et al*., 2004). Strikingly, both GFP-CLIC4 and endogenous CLIC4 showed perfect colocalization with DsRed-tagged NHERF2 in a polarized, restricted zone along the plasma membrane (Figure 4, B and C). Furthermore, endogenous CLIC4 colocalized with both the HA-tagged $LPA_2$ receptor and DsRed-NHERF2 (Figure 4C). In the second approach, we coexpressed the GFP-tagged $LPA_1$ receptor and CLIC4-mCherry in HEK293 cells. LPA stimulation caused a rapid accumulation of CLIC4-mCherry at $LPA_1$-containing subdomains at the plasma membrane (Figure 4D and Supplemental Video 3). Finally, we applied a precisely confined stream of TRP ligand (generated through an application and a suction micropipette) to single GFP-CLIC4–expressing cells. This induced a rapid, highly polarized accumulation of CLIC4 exclusively at the agonist-exposed area of the plasma membrane (Figure 4E and Supplemental Video 4). Together, these results strongly suggest that CLIC4 is targeted specifically to those plasma membrane domains that contain RhoA-activating GPCRs in complex with their scaffolds.

### *Mutational Analysis of CLIC4 Recruitment: Identification of Critical Residues Based on Comparison with the "GST-Fold"*

We next examined which residues are essential for CLIC4 recruitment, taking into account the structural similarity of CLIC4 to omega-GST (Board *et al*., 2000; Littler *et al*., 2005). Mammalian GST classes have the same basic structural fold. This GST-fold consists of two domains: an N-terminal thioredoxin-like domain that binds glutathione followed by an

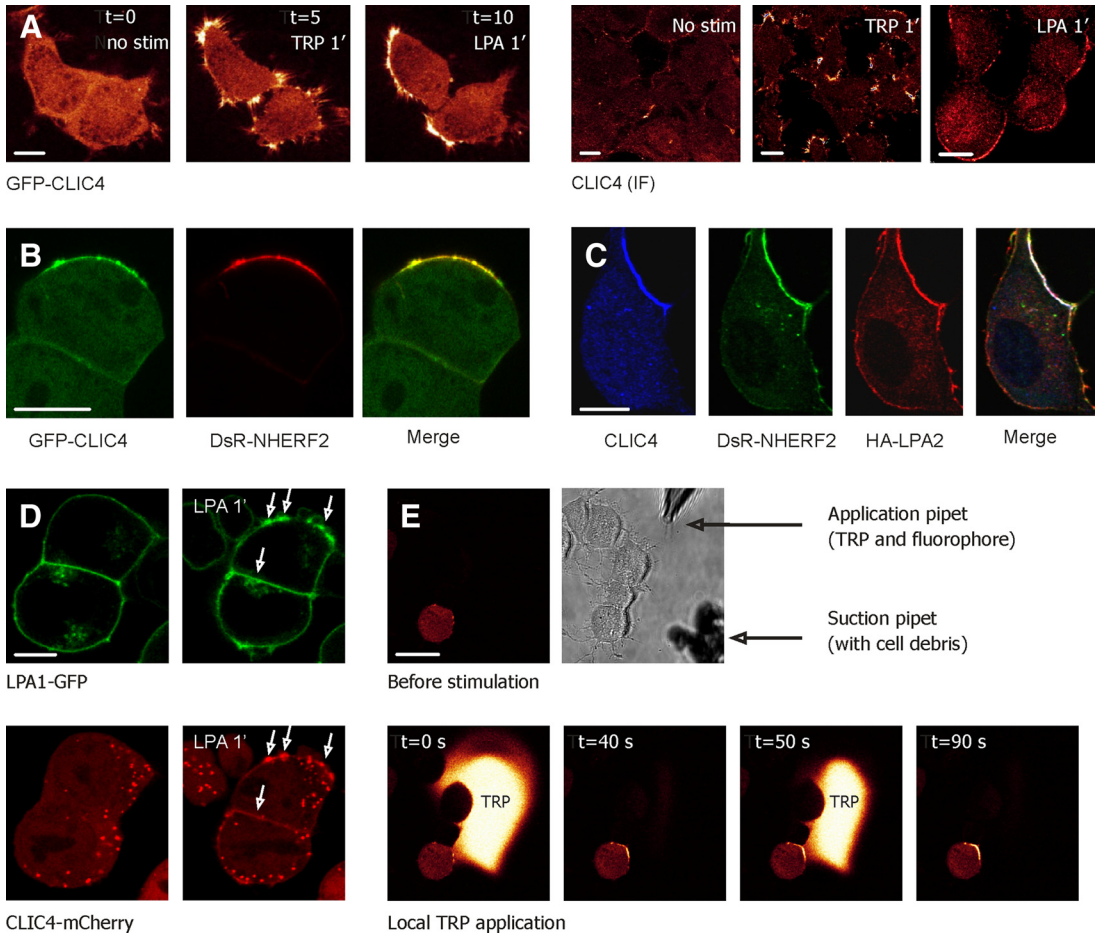
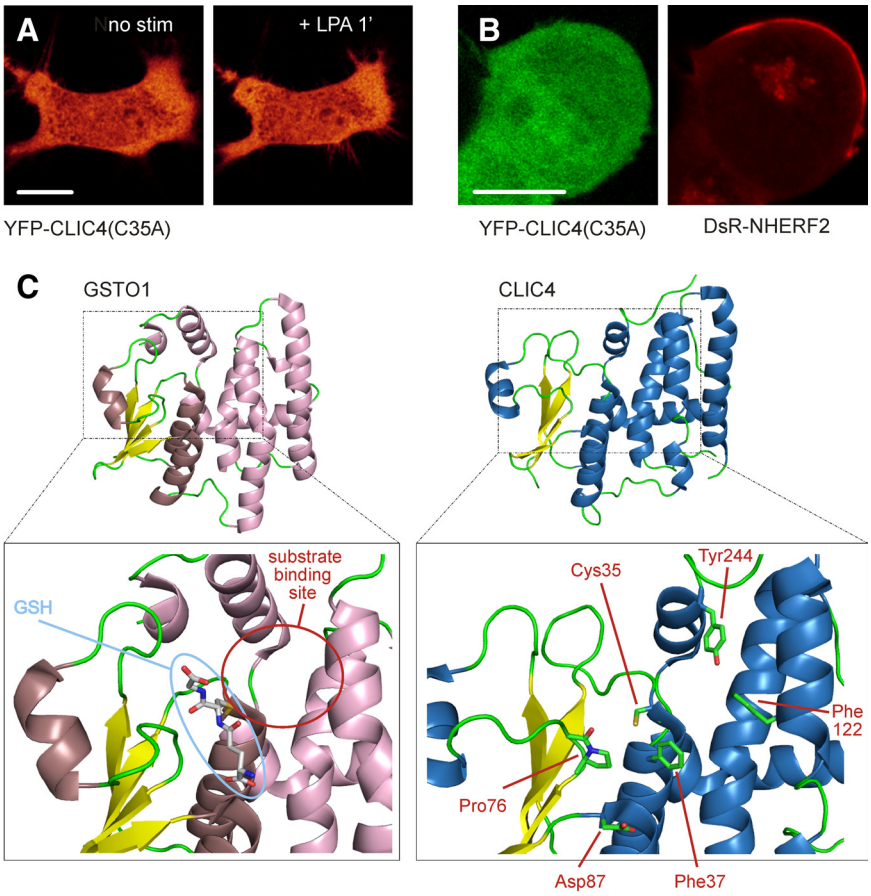



**Figure 4.** CLIC4 translocation toward activated GPCRs and NHERF2. (A) Differential distribution of translocated GFP-CLIC4 in the same cell. N1E-115 cells were first stimulated with TRP (50 $\mu$M) and thereafter with LPA (1 $\mu$M). Also see Supplemental Video 2. Right, differential recruitment of endogenous CLIC4 by LPA and TRP. Bars, 10 $\mu$m. (B) Colocalization of GFP-CLIC4 and DsRed-NHERF2 at polarized regions of the plasma membrane in N1E-115 cells. Bar, 10 $\mu$m. (C) Triple colocalization of endogenous CLIC4 (Alexa488-conjugated secondary antibody; shown in blue), DsRed-NHERF2 (shown in green), and HA-LPA2 (Alexa633-conjugated secondary antibody; shown in red). Bar, 10 $\mu$m. (D) CLIC4 translocates to activated LPA receptor complexes. Pictures taken from time-lapse movies showing LPA1-GFP and CLIC4-mCherry before and after LPA stimulation. Arrows indicate plasma membrane regions where LPA1-GFP and CLIC4-mCherry transiently colocalize after LPA addition (also see Supplemental Video 3). Bar, 10 $\mu$m. (E) CLIC4 accumulates selectively in ligand-exposed membrane domains. TRP was locally applied by combining an application and a suction pipette. Besides TRP (3 $\mu$M), the application pipette contained calcium orange (0.2 mM) to continuously monitor the flux between both pipettes. After verifying the gradient steepness of the TRP/dye mix, a selected cell was positioned near the pipettes and GFP-CLIC4 was imaged during local TRP application. Top, fluorescence and transmission images before ligand application. Arrows indicate pipettes (cell debris was attracted by the suction pipette). Bottom, at t = 0, a brief pulse of TRP reaches a small area of the plasma membrane. At t = 40 s, GFP-CLIC4 accumulates selectively at the TRP-exposed area of the plasma membrane. At t = 50 s, a second TRP pulse is applied. At t = 90 s, further accumulation of GFP-CLIC4 is observed. Also see Supplemental Video 4. Pictures are representative of four independent experiments. Bar, 20 $\mu$m.

all-helical C-terminal domain that binds the substrates to which glutathione is conjoined. The omega-GSTs are unusual, however, in that they have a unique range of catalytic activities compared with other GSTs and typically contain a reactive cysteine in their glutathione-binding site that can form a mixed disulfide bond (Whitbread *et al.*, 2005); this cysteine is conserved in the mammalian CLICs (residue Cys35 in CLIC4; Littler *et al.*, 2005). Under reducing conditions, as normally found in the cytosol, the CLICs exhibit very low affinity for glutathione and no substrates are known to which they display transferase activity. The conserved cysteine is a potential site for oxidative regulation (Littler *et al.*, 2004. 2005). However, application of either an oxidative burst (1 mM $H_2O_2$) or an antioxidant (*N*-acetylcysteine; 5 mM) did not detectably modulate the translocation behavior of CLIC4 (data not shown), indicating that CLIC4 recruitment is not subject to redox regulation.

To determine the importance of residue Cys35, we generated the CLIC4(C35A) mutant. As shown in Figure 5A, YFP-tagged CLIC4(C35A) failed to translocate to the plasma membrane upon receptor stimulation. Likewise, the colocalization with DsRed-NHERF2 was completely lost (Figure 5B). In contrast, mutation of two other conserved cysteines, Cys189 and Cys234, did not affect CLIC4 translocation (Figure 5D). So, if CLIC4 maintains an as-yet-undetected enzymatic activity required for translocation, homology to the omega-GSTs suggests that Cys35 serves as active site.

We next selected residues whose equivalents make up the glutathione-binding site in GSTs. Superposition of the CLIC4 and omega-GST structures (Board *et al.*, 2000; Littler




D

| YFP-CLIC4 | Agonist-induced translocation | NHERF2 colocalization |
|---|---|---|
| wt | + | + |
| C35A | - | - |
| F37D | - | - |
| D87A | - | - |
| P76A | - | - |
| F122R | - | - |
| Y244A | - | - |
| C189S | + | + |
| C234S | + | + |

**Figure 5.** Mutational analysis of CLIC4 translocation. (A and B) Mutation of residue Cys35 (YFP-CLIC4(C35A)) abolishes LPA-induced translocation (A) and colocalization with DsRed-NHERF2 (B). Bars, 10 $\mu$m. (C) Structural comparison of omega-GST (GSTO1) and CLIC4. The indicated residues were mutated, based on their equivalence to the residues that mediate the binding of glutathione and secondary substrates in GSTs. Results are summarized in D. See text for details.

*et al*., 2005) revealed several conserved residues that may be important, namely, Phe37, Pro76, and Asp87 (Figure 5C). In GSTs, mutating the Phe37 equivalent into an aspartate would add a repulsive interaction toward the carboxyl group of glutathione's $\gamma$-glutamate, mutating the equivalent of Pro76 is known to reduce catalytic activity and conformation stability (Nathaniel *et al*., 2003), while mutating the equivalent of Asp87 (often a Glu or Gln) affects substrate binding and catalysis (Manoharan *et al*., 1992). Mutating any of these three residues abolished agonist-induced translocation of YFP-CLIC4 as well as its colocalization with DsRed-NHERF2 (summarized in Figure 5D).

GSTs also bind a second substrate, namely, the xenobiotic compound to which glutathione is conjugated. This second binding site is less conserved and therefore harder to define. Because any such substrate must lie next to glutathione for conjugation, distance constraints reduce the number of residues that need to be considered. Phe122 and Tyr244 are strongly conserved among the CLICs and occur at positions equivalent to the secondary substrate binding site of the GSTs (Figure 5C). We therefore generated mutants CLIC4(F122R) and CLIC4(Y244A) and expressed them in N1E-115 and HEK293 cells. Both mutants failed to undergo agonist-induced translocation; neither did they colo-

calize with coexpressed DsRed-NHERF2 (Figure 5D). Collectively, these results suggest that the substrate-binding features of the omega-GSTs have been conserved in CLIC4, along with the fold itself, and that binding of an as yet unknown substrate is essential for CLIC4 to translocate upon receptor stimulation.

## DISCUSSION

The present study defines CLIC4 as a novel player in RhoA signaling. Cytosolic CLIC4 rapidly responds to RhoA activation by translocating to plasma membrane micropatches containing activated $G_{13}$-coupled receptors in complex with their scaffolds, such as the $LPA_2$–NHERF2 complex. We find that CLIC4 recruitment requires F-actin polymerization but is independent of RhoA's major downstream effector ROCK. Membrane-targeted CLIC4 does not seem to induce or modulate plasma membrane $Cl^-$ channel activity, because knockdown of CLIC4 or blocking CLIC4 recruitment (using latrunculin A) has no detectable effect on transmembrane $Cl^-$ currents as measured by patch clamp. The latter finding argues against the notion that CLIC4 is a component of a plasma membrane $Cl^-$ channel (Proutski *et al*., 2002). However, we cannot exclude the possibility that membrane-targeted CLIC4 may influence $Cl^-$ movements in vesicles just underneath the plasma membrane, which escapes detection by patch clamp.

Through mutational analysis, we have identified six conserved residues, including the reactive Cys35, that are essential for CLIC4 to respond to RhoA activation and whose equivalents are critical for substrate processing in GSTs. Although CLIC4 lacks classical GST activity, the more related omega-class GSTs (characterized by an active-site Cys residue) show various unique thioltransferase and reductase activities as well as stress response properties that are still incompletely characterized (Whitbread *et al*., 2005). This leads us to suggest that CLIC4 may function as a transferase that uses Cys35 as a catalytic residue to bind and process an as yet unknown substrate associated with RhoA activity.

What could be the function of CLIC4 downstream of activated RhoA? Some hints may come from the finding that *Clic4* knockout mice suffer from impaired angiogenesis and that CLIC4 is essential for vacuole formation during endothelial tubulogenesis (Ulmasov *et al*., 2009). This is reminiscent of the defective tubulogenesis of the excretory canal in *C. elegans* lacking the CLIC homologue EXC-4 (Berry *et al*., 2003). Tubulogenesis occurs through the intracellular fusion and subsequent exocytosis of vacuoles (Kamei *et al*., 2006), which in turn is regulated by the exocyst multiprotein complex (Munson and Novick, 2006; Wu *et al*., 2008). The exocyst is also involved in regulating F-actin polymerization (Zuo *et al*., 2006). Interestingly, it has been shown that RhoA can interact, either directly or indirectly, with the exocyst complex and thereby may control tubulogenesis (Rogers *et al*., 2003). Given the RhoA-CLIC4 connection reported here, it will be interesting to explore a possible role of CLIC4 in RhoA-dependent functions of the exocyst, not only in vesicle fusion and membrane expansion but also in actin dynamics.

Furthermore, the present findings warrant further research into the specific roles of the $G_{12,13}$-RhoA pathway in vivo (Worzfeld *et al*., 2008). For example, are the severe angiogenic defects observed in $G\alpha_{13}$ knockout mice (Offermanns *et al*., 1997) attributable, at least in part, to loss of CLIC4 function? Conversely, do *Clic4* knockout mice show alterations in $G_{12/13}$-RhoA-mediated activities such as blood clotting, vascular smooth muscle contraction, and salt-induced hypertension (Moers *et al*., 2003; Wirth *et al*., 2008)? Our foremost challenge, however, is to identify the specific binding partners and putative substrate(s) of CLIC4.

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