Chloride intracellular channel protein CLIC4 (p64H1) binds directly to brain dynamin I in a complex containing actin, tubulin and 14-3-3 isoforms

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Mammalian chloride intracellular channel (CLIC) (p64-related) proteins are widely expressed, with an unusual dual localization as both soluble and integral membrane proteins. The molecular basis for their cellular localization and ion channel activity remains unclear. To help in addressing these problems, we identified novel rat brain CLIC4 (p64H1) binding partners by affinity chromatography, mass spectrometric analysis and microsequencing. Brain CLIC4 binds dynamin I, α-tubulin, β-actin, creatine kinase and two 14-3-3 isoforms; the interactions are confirmed *in vivo* by immunoprecipitation. Gel overlay and reverse pull-down assays indicate that the binding of CLIC4 to dynamin I and 14-3-3ζ is direct. In HEK-293 cells, biochemical and immunofluorescence analyses show partial co-localization of recombinant CLIC4 with caveolin and with functional caveolae, which is consistent with a dynamin-associated role for CLIC4 in caveolar endocytosis. We speculate that brain CLIC4 might be involved in the dynamics of neuronal plasma membrane microdomains (micropatches) containing caveolin-like proteins and might also have other cellular roles related to membrane trafficking. Our results provide the basis for new hypotheses concerning novel ways in which CLIC proteins might be associated with cell membrane remodelling, the control of cell shape, and anion channel activity.

Key words: anion channels, caveolin, endocytosis, HEK-293 cells.

INTRODUCTION

Eukaryotic cells contain functional channels in the endoplasmic reticulum and sarcoplasmic reticulum [1–3]; specific ion channels have also been identified in the inner nuclear membrane [4], mitochondrial membranes [5,6], Golgi membranes [7,8] and secretory vesicles [9–11]. Some of these channels are involved in the mobilization of intracellular Ca^{2+} [1,2] and the transport of metabolites [5]. However, the molecular identities and cellular roles of many intracellular channels remain to be discovered. These include the 'charge-compensating' anion channels found in some acidic vesicles containing proton pumps [12]. In addition, just as plasma membrane ion channels help to regulate cell volume [13,14], intracellular ion channels might regulate the volume of intracellular organelles or might store channels for rapid on-demand incorporation into, and removal from, the plasma membrane [14].

The bovine renal protein p64 [12] was the first member of a new family of putative intracellular anion channel proteins, many of which are now collectively called chloride intracellular channel (CLIC) proteins, to be identified at the molecular level [15]. CLIC4, originally named p64H1 because it was the first homologue of p64 to be discovered [16], is a 28.6 kDa protein corresponding to a cDNA cloned from rat brain. CLIC4 mRNA is widely expressed in neuronal and non-neuronal tissues; cells overexpressing recombinant CLIC4 contain intracellular anion channel activity that is absent from mock-transfected cells [17]. Indirect immunofluorescence, cell fractionation and immunoblotting studies have localized native and recombinant CLIC4 proteins both to the cytosol and to intracellular membranes. A similar, very unusual, dual localization is characteristic of many CLIC and p64-related proteins [18–24].

The CLIC/p64 superfamily has recently grown to include p64 itself, the p64-like protein parchorin [24] and five CLIC proteins. Human CLIC1 was identified as a putative nuclear membrane Cl^- channel [19]. Human CLIC2 is a hypothetical protein encoded by the putative gene XAP121 [25]. The cDNA encoding human CLIC3, postulated like CLIC1 to be a nuclear membrane Cl^- channel protein, was cloned during a yeast two-hybrid screen [20] for partners interacting with the C-terminal domain of an atypical mitogen-activated protein kinase, ERK-7. CLIC4 cDNA, which was cloned from rat brain by using reverse-transcriptase-mediated PCR approaches based on the sequence of p64 [16,17], has both human [21,22] and mouse [23] homologues. The mouse protein was named mtCLIC because it localized in part to mitochondria. Finally, a CLIC5 cDNA has been cloned from human placenta [26].

The membrane locations of CLIC4 are diverse. For example, when recombinant rat brain CLIC4 is transiently expressed in rat hippocampal HT-4 cells, it is directed primarily to the endoplasmic reticulum [17]. In contrast, whereas human CLIC4 partly co-localizes with caveolae in a pancreatic cell line [22], the mouse homologue of CLIC4 (mtCLIC) is markedly, but not exclusively, localized to mitochondria [23]. Rat brain CLIC4 has also been localized to large dense core vesicles in neurosecretory cells [21]. Such a protean distribution could reflect a shuttling of CLIC4 between the cytoplasm and different endomembrane systems or an involvement of the protein in a widespread cell biological process such as membrane trafficking or vesicle transport. The structural basis for its 'multisite' targeting might be relevant to the putative channel function of CLIC4.

There are several alternative ways in which CLIC proteins could be linked to intracellular ion channel activity. They could be channel-forming proteins, they could activate endogenous ion

Abbreviations used: cd, cytoplasmic domain; CLIC, chloride intracellular channel; CT, cholera toxin; ECL, enhanced chemiluminescence; GST, glutathione S-transferase; IAA, indanoyloxyacetic acid; TRITC, tetramethylrhodamine isothiocyanate.

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channels (either directly or indirectly) or they could perform both functions. The 'minimal ion channel' hypothesis has been directly tested for CLIC1, which forms channels after being incorporated, as a pure recombinant protein, into planar bilayers [27]. Although it has yet to be shown that this channel activity *in vitro* corresponds to an endogenous ion channel, it is clearly possible that CLIC1 and other CLIC proteins might have a direct role as intracellular ion channels. An alternative hypothesis is that some or all of the CLIC proteins are anion channel regulators or components, rather than (or as well as) ion channels themselves. This hypothesis draws on the increasing awareness that some putative ion channel proteins activate endogenous ion channels [28]. If this is true for CLIC4, it must bind to specific partners in the cell. Here we set out to identify protein partners to help to define the cellular roles of CLIC4, and possibly other CLIC proteins, in more detail.

Using a glutathione S-transferase (GST) 'pull-down' assay, we describe the isolation of a novel protein complex associated with the cytoplasmic domain of brain CLIC4. The complex contains dynamin I, two 14-3-3 isoforms, β-actin, α-tubulin and creatine kinase. We confirm by immunoprecipitation that CLIC4 interacts with the proteins *in vivo* and we demonstrate that the interactions of CLIC4 with (at least) dynamin I and 14-3-3ζ are direct. The association of CLIC4 with dynamin and cytoskeletal proteins might be relevant to many cellular functions. Our results support the suggestion that CLIC4 is involved in caveolar endocytosis in HEK-293 cells and, by implication, in the function of caveolae-like domains in neurons. This does not exclude a role for neuronal CLIC4 in synaptic vesicle endocytosis or in other cellular events. Finally, in the light of these new findings, we suggest indirect ways in which CLIC4 could be linked to intracellular anion channel activity.

MATERIALS AND METHODS

CLIC4, 14:3:3ζ and dynamin I GST fusion proteins

A cDNA encoding the cytoplasmic domain (CLIC4cd) corresponding to residues 60–253 of CLIC4 (p64H1 [16,17]) was amplified from rat brain *p64H1* cDNA by PCR, using the oligonucleotides CGCGGATCCAAAAGGAAGCCTGCACATCTGCAGAACTTGGC and ATACCGCTCGAGCTACTTGGTAAGTCTCTTGGCGACG. The insert was cloned into pGEX-4T1 (Pharmacia) by using engineered 5′ *Bam*H1 sites and 3′ *Xho*I sites (underlined), then sequenced. The recombinant plasmid was transformed into *Escherichia coli* HMS174 (DE3) and the bacteria were grown overnight at 37 °C with shaking (250 rev./min) in Luria–Bertani medium containing 50 μg/ml ampicillin, diluted 1:9 into M9MM medium containing 50 μg/ml ampicillin and maintained at 37 °C with shaking until D_{600} reached 0.8. Protein expression was induced by the addition of 0.1 mM isopropyl β-D-thiogalactoside; the cells were incubated for a further 4 h at 25 °C with shaking. The cells were then pelleted at 8000 ***g*** for 15 min at 4 °C; a crude cell lysate was prepared by sonication (five times, 30 s each; MSE SoniProbe sonicator at maximum power) in PBS containing protease inhibitor cocktail tablets (Boehringer). Where required, CLIC4cd was isolated by coupling the GST fusion protein to glutathione–Sepharose beads (Pharmacia), followed by extensive washing and finally digestion with thrombin (Sigma) for 18 h at 4 °C. Rat brain *dyn*-1 cDNA in psVL (Pharmacia) was a gift from Richard Vallee (University of Massachusetts Medical School, Worcester, MA, U.S.A.); we amplified a cDNA corresponding to full-length *dynamin I* by PCR with the oligonucleotides CCGGAATTCATGGGCAACCGCGGCATGGAAGACCTCATC and CCGCTCGAGTCAGGGGTCACTGATAGTGATTCTGGGGAC. We incorporated a 5′ *Eco*RI site and a 3′ *Xho*I site (both underlined) to facilitate cloning into pGEX-4T1 (Pharmacia). 14-3-3ζ cDNA in pGEX-2T1 was a gift from Thierry Dubois (University of Edinburgh, Edinburgh, U.K.). Dynamin I and 14-3-3ζ GST fusion proteins were expressed in *E. coli* HMS174 (DE3), under the conditions outlined earlier for CLIC4.

Affinity purification

For affinity purification of CLIC4-binding proteins, we mixed 100 ml of rat brain cytosol (500 mg of protein) in 0.32 M sucrose (prepared as described previously [17]) with an equal volume of binding buffer [50 mM Tris/HCl (pH 7.4)/50 mM NaCl/1 mM EDTA/1 mM EGTA/5% (v/v) glycerol] containing protease inhibitors, and precleared it by incubation for 3 h at 4 °C with GST attached to glutathione beads. The precleared cytosol was incubated overnight at 4 °C with CLIC4cd GST fusion protein attached to glutathione beads (35 mg of protein/ml of bead slurry) with continuous gentle mixing. The suspension was placed in a 20 ml disposable column; the beads were washed with a 10-fold excess of binding buffer. The final 10 ml of washings was retained. Proteins that remained bound to the immobilized CLIC4cd were eluted by 10 ml of 1 M NaCl in binding buffer, followed by 10 ml of 2 M NaCl in binding buffer. The final 10 ml of washings and the eluted protein fractions were dialysed against 10 mM Tris/HCl, pH 8.4, at 4 °C, reduced to a volume of 100 μl with Centricon (Amicon) concentrators (10 kDa molecular mass cut-off) and subjected to SDS/PAGE in a linear gradient of 6–15% (w/v) polyacrylamide. For 'reverse' pull-down assays, we incubated CLIC4cd (300 μg of protein in 1 ml of binding buffer) overnight at 4 °C with 1 mg of 14-3-3ζ–GST fusion protein attached to glutathione beads. After washing of the column as described earlier, bound proteins were eluted with 10 ml of 2 M NaCl in binding buffer, followed by dialysis, microconcentration and SDS/PAGE as described previously.

MS

Protein bands in the complex eluted from immobilized CLIC4cd were identified by staining SDS/PAGE gels for 60 min with GelCode (Pierce); the gels were then destained overnight in a large volume of cold distilled water. After excision of the band and in-gel digestion with trypsin (Promega), the resulting peptides were concentrated in a SpeedVac vacuum centrifuge, desalted by microbore reverse-phase chromatography (C_{18}) and analysed on a Thermoquest LCQ ion-trap mass spectrometer in nanospray mode. Data from MS/MS experiments were analysed with Sequest software.

Antibody production, immunoblotting and immunoprecipitation

Rabbit antiserum was raised (Diagnostics Scotland) against recombinant CLIC4cd emulsified with TiterMax Gold (CytRx); this was used with or without affinity purification on immobilized CLIC4 with similar results. Western blot analysis was performed as described previously [17]. For immunoprecipitations, samples of cytosol were precleared by the addition of Protein G–Sepharose beads (Sigma) as a 50% (v/v) slurry in PBS (100 μl per ml of lysate) with incubation at 4 °C for 30 min, followed by centrifugation at 14000 ***g*** for 3 min to remove the beads. The precleared cytosol was mixed with anti-CLIC4 antiserum at the specified dilutions and incubated at 4 °C for 60 min with

gentle rolling. The protein–antibody complex was captured by the addition of 5 % (v/v) of a 50 % (v/v) Protein G–agarose slurry and the mixture was gently mixed (by rolling) for at least 60 min at 4 °C. The beads and captured immunocomplex were collected by centrifugation (14000 ***g*** for 5 min) at 4 °C, washed three times with PBS and resuspended in SDS/PAGE loading buffer. Complexes were subjected to SDS/PAGE [10 % or 12 % (w/v) gel] and analysed by Western blotting with enhanced chemiluminescence (ECL®; Amersham) detection, by using the antibodies listed in the Results section and the relevant Figure legends. Densitometric analysis was performed with NIH Image; molecular mass markers were from Sigma and Bio-Rad.

Gel-overlay assays

Gel-overlay assays were performed with the technique described by Witke et al. [29]. In brief, brain cytosol proteins were subjected to SDS/PAGE [10 % (w/v) gel] and protein bands were transferred to nitrocellulose-C (Amersham) membranes. The membranes were rinsed briefly in NCP buffer comprising 20 mM Tris/HCl, pH 8.4, 150 mM NaCl, 0.05 % (v/v) Tween 20 and 0.02 % (w/v) NaN_3, then incubated overnight at 4 °C in NCP buffer containing 5 % (v/v) foetal bovine serum and 0.5 mM dithiothreitol. The membranes were then incubated with 30 μg/ml recombinant CLIC4cd for 4 h at 4 °C and washed three times (10 min each) with NCP buffer. After incubation with anti-CLIC4 antiserum and further washing with NCP buffer, labelled protein bands were revealed with horseradish-peroxidase-coupled secondary antibodies and ECL detection (Amersham).

Actin spin-down assays

G-actin was prepared from acetone-dried powder of rabbit skeletal muscle (α-actin [30]) or purchased from 'Cytoskeleton' as purified platelet actin (a mixture of β-actin and γ-actin). The actins were polymerized in the presence or absence of CLIC4 in a buffer containing 50 mM KCl, 0.2 mM ATP, 0.5 mM dithiothreitol and 0.2 mM $CaCl_2$, with or without 1 mM EGTA and with or without 1 mM $MgCl_2$. The solutions were buffered to a pH of 6.5 with 10 mM imidazole/HCl or to a pH of 8.0 with 10 mM Tris/HCl. Care was taken to maintain the same salt concentration in every assay. After 60 min of polymerization reactions in a total volume of 0.5 ml, F-actin was separated by centrifugation (300000 ***g*** for 20 min; Beckman TL-100). The supernatant, containing non-polymerized G-actin, was carefully removed and mixed with an equal volume of 2 × SDS/PAGE sample buffer. The pellets were resuspended in 0.5 ml of polymerization buffer and placed in fresh tubes. The original tubes were washed with 0.5 ml of 2 × SDS/PAGE sample buffer; the washings were combined with the resuspended pellets. Equal volumes of the samples (pellets and supernatants) were analysed by SDS/PAGE. The actin-binding protein cofilin (a gift from Sutherland Maciver, University of Edinburgh, Edinburgh, U.K.) was used as a positive control.

Expression and detection of proteins in mammalian cells

Full-length *CLIC4* and *dynamin I* cDNA species were cloned into pcDNA3.1zeo (Invitrogen) and transfected into HEK-293 cells maintained in antibiotic-free Dulbecco's modified Eagle's medium supplemented with 10 % (v/v) foetal bovine serum (Gibco), by using LIPOFECTAMINE (Gibco) in accordance with the manufacturer's instructions. CLIC4-transfected cells were passaged in the presence of 0.2 mg/ml Zeomycin (Zeocin™; Invitrogen) to select stably transfected cell lines, having determined that this antibiotic concentration was lethal for non-transfected, control cells. Expressed proteins were detected by immunoblotting (see above) after cell fractionation in the presence of protease inhibitors [17] or by indirect immunofluorescence [17] of fixed cells, with the use of our anti-CLIC4 antiserum or a goat polyclonal anti-(dynamin I) antibody (Santa Cruz). Anti-caveolin 1 monoclonal antibody was from Transduction Laboratories; the secondary antibodies used for immunofluorescence were goat anti-rabbit FITC- or tetramethylrhodamine isothiocyanate (TRITC)-coupled IgGs, rabbit anti-goat TRITC-coupled IgG or goat anti-mouse TRITC-coupled IgG (all from Sigma), as appropriate. In some experiments, actin was post-stained with TRITC-coupled phalloidin (Sigma); alternatively the uptake of TRITC-labelled transferrin (Molecular Probes) or FITC-labelled cholera toxin (CT) B subunit (Sigma) was monitored by adding the appropriate protein before the cells were washed and fixed. Confocal microscopy was performed with a Leica confocal fluorescence microscope; images were displayed and analysed with Leica and Bitplane software.

RESULTS

Isolation of CLIC4-binding proteins from rat brain cytosol

CLIC4 exists in cells as both a soluble and a membrane-associated protein [16,17,21–23]. Proteolytic digestion of membrane-associated rat brain CLIC4 translated *in vitro* in the presence of microsomes showed the membrane-associated form to be a single-pass integral membrane protein with a small N-terminal luminal domain and a large cytoplasmic domain [17]. These findings are consistent with uniformly strong structural predictions of a single transmembrane domain encompassing residues 38–59 and the presence of multiple consensus phosphorylation sites in the C-terminal domain, at least some of which are functional [17]. As expected, the presumed cytoplasmic domain of CLIC4, corresponding to the final 194 residues and

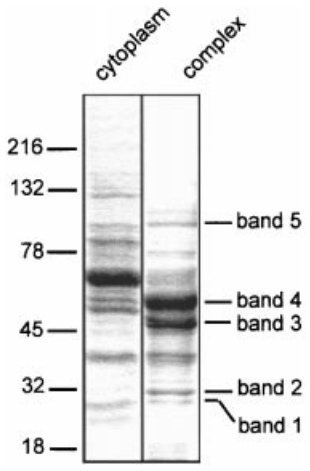

Figure 1 Affinity purification of CLIC4-binding proteins from rat brain cytosol

Rat brain cytosol (100 ml) containing 500 mg of protein was subjected to affinity chromatography with immobilized CLIC4cd. After extensive washing, CLIC4-bound proteins were eluted by a step gradient of 1 M NaCl, dialysed to remove the salt and concentrated to 100 μl. Affinity-purified proteins in 20 μl (20%) of the CLIC4-bound protein complex are compared with proteins present in 5 μl (25 μg) of the original unfractionated rat brain cytoplasmic protein, by using SDS/PAGE with 6–15% (w/v) gradient gels (stained with GelCode). The protein bands labelled 1–5 were subjected to successful in-gel digestion with trypsin followed by MS analysis (Table 1). The positions of molecular mass markers are indicated (in kDa) at the left.

Table 1 Rat brain CLIC4-binding proteins identified by MS

Peptide fragments from gel bands 1–5 (see Figure 1) were identified by exact mass analysis and database searching and by MS/MS sequencing in nanospray ion-trap MS. Confirmatory BLAST searches revealed exact correspondences to sequences in the listed *Rattus norvegicus* proteins. Note that band 3 contained both β-actin and creatine kinase β-chain, which have similar molecular masses.

Gel band	Peptide mass (Da)	MS/MS sequence	Protein	Accession number	Molecular mass (calculated) (Da)
1	1418.7	DICNDVLSLLEK	14-3-3ζ	JC5232	27800
	2041.0	GIVDQSQQAYQEAFEISK		JC5232	
2	1476.7	LICCDILDVLDK	14-3-3ϵ	AAC52676	29120
	2087.9	AAFDDAIAELDTLSEESYK		AAC52676	
3	1303.7	VLTPELYAELR	Creatine kinase β-chain	AAA40933	40623
	1557.8	FCTGLTQIETLFK		AAA40933	
	1863.9	LGFSEVEQVQMVVDGVK		AAA40933	
	1964.9	GTGGVDTAAVGGVFDVSNADR		AAA40933	
	1790.9	SYELPDGQVITIGNER	β-actin	ATRTC	41749
	2231.1	DLYANTVLSGGTTMYPGIADR		ATRTC	
4	1598.7	TIQFVDWCPTGFK	α-tubulin	P02551	50134
	2007.9	TIGGGDDSFNTFFSETGAGK		P02551	
5	1609.8	TGLFTPDLAFEATVK	Dynamin I	P21575	95849
	1709.9	VPVGDQPPDIEFQIR		P21575	
	1782.9	ALLQMVQQFAVDFEK		P21575	
	1867.8	IEGSGDQIDTYELSGGAR		P21575	

comprising approx. 75% of the protein, was soluble after cleavage from GST (results not shown). To isolate proteins interacting with brain CLIC4cd, and with soluble CLIC4, we used the immobilized CLIC4 GST fusion protein to 'pull down' interacting proteins from rat brain cytosol.

A typical result from such a pull-down or affinity purification procedure is shown in Figure 1. The same results were obtained in four experiments with three independent brain cytosol preparations. After in-gel digestion with trypsin and microbore reverse-phase HPLC, we succeeded in identifying six proteins in the CLIC4 complex by MS/MS analysis (Figure 1 and Table 1). These included the ζ and ϵ isoforms of 14-3-3, β-actin, creatine kinase, dynamin I and α-tubulin. We were unable to obtain fragment masses or sequences from any of the other gel bands, from which we obtained insufficient protein after in-gel digestion.

Immunoprecipitation of CLIC4-associated proteins

Although dynamin I and the other identified proteins bind (directly or indirectly) to CLIC4 *in vitro*, the proteins might be located in different cellular compartments, and might be unable to associate *in vivo*. We therefore performed immunoprecipitation studies to verify interactions *in vivo*. We confirmed that dynamin I was immunoprecipitated from rat brain cytosol by anti-CLIC4 rabbit polyclonal antiserum and also by affinity-purified anti-CLIC4 antibody (Figure 2a). The antiserum was most effective at a dilution of 1:100. We also confirmed that the anti-CLIC4 antiserum immunoprecipitated β-actin, α-tubulin and 14-3-3ζ from rat brain cytosol at this dilution (Figure 2b). A specific non-rabbit anti-(14-3-3ϵ) antibody was not available. Treatments involving detergents (including Triton-X100 or CHAPS-containing buffers) substantially decreased the binding of CLIC4 to its protein partners; we were unable to extend immunoprecipitation studies to membrane-bound proteins.

Dynamin I and 14-3-3ζ bind directly to CLIC4

The proteins in the CLIC4 complex might be bound to CLIC4 directly or indirectly. To help determine which protein or proteins in the complex were bound directly to CLIC4, we performed gel-overlay assays as described in the Materials and methods section. Both dynamin I and 14-3-3ζ bound directly to CLIC4 without any requirement for a linker or adapter molecule (Figure 3). We could not detect direct binding of CLIC4 to α-tubulin, β-actin or creatine kinase. We conclude that either (1) these proteins bind indirectly to CLIC4 (e.g. via dynamin I or a 14-3-3 adapter complex) or (2) the relevant binding sites are disrupted after the gel electrophoresis and protein transfer steps.

We performed 'reverse' pull-down assays to confirm a direct interaction between CLIC4 and immobilized 14-3-3ζ. A relatively small amount of CLIC4cd appeared in the final washings, whereas a substantial proportion of the added protein was specifically bound to 14-3-3ζ and was released by elution at high salt concentration (Figure 4a). To examine the interaction in more detail, we performed CLIC4 binding assays in the presence and absence of synthetic peptides corresponding to the Raf 14-3-3 binding sequence LSGRGRST<u>S</u>TPNVHMV (single-letter amino acid codes), where the underlined serine residue was either non-phosphorylated or phosphorylated. Both Raf peptides, especially the phosphopeptide, decreased the binding of CLIC4 to 14-3-3 ζ (Figure 4b). CLIC4 binding was unaffected by an unrelated control peptide, NVVGARRSSWRVISSIEQKT (residues 51–70 of the γ isoform of 14-3-3), and the phosphopeptide decreased CLIC4 binding significantly more than the non-phosphorylated peptide (Figure 4c). Unlike with the 14-3-3 proteins, there is no evidence that recombinant dynamin I can refold when expressed as a GST fusion protein, precluding similar reverse binding experiments.

Interaction of CLIC4 with actin

The association of brain CLIC4 with actin recalls the isolation of human placental CLIC5 within a protein complex binding to the free C-terminus of ezrin [26]. The ezrin complex also included actin; it was suggested that ezrin might enhance the binding of CLIC5 to actin, although there was no firm evidence for a direct interaction between the two proteins. Actin–protein complexes are known to be difficult to reassemble *in vitro*; the apparent lack of any direct interaction in gel-overlay assays does not exclude a direct interaction *in vivo*. In an attempt to examine this potentially important interaction in more detail, we performed actin spin-

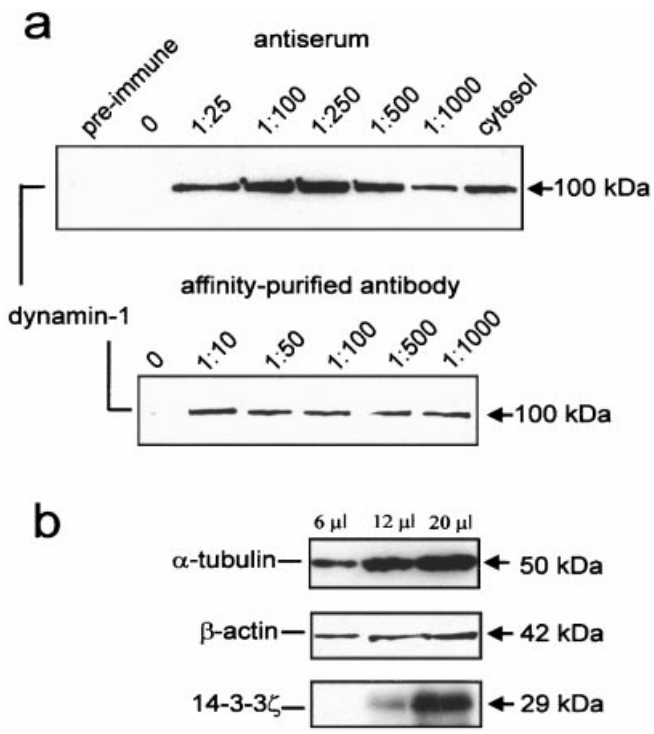

Figure 2 Immunoprecipitation of CLIC4-binding proteins from rat brain cytosol

Dynamin I, β-actin, α-tubulin and 14-3-3ζ were immunoprecipitated by rabbit polyclonal anti-CLIC4 antibodies from 1 ml aliquots of rat brain cytosol (5 mg of total protein). The captured immunocomplexes were solubilized in 60 μl of SDS/PAGE sample buffer; aliquots were subjected to SDS/PAGE [12% (w/v) gel] and immunoblotting. No signals were obtained in immunoprecipitation controls from which either cytosol or anti-CLIC4 antibody was omitted. (**a**) Upper panel: dose–response for the immunoprecipitation of dynamin I from individual reactions performed with 1 ml of cytoplasm (5 mg of protein). Rabbit anti-CLIC4 antiserum was used at the indicated dilutions; each lane contained 20 μl of immunocomplex in sample buffer, corresponding to 33% of the total immunoprecipitated protein, with 5 μg of cytosolic protein in the right-hand lane. Lower panel: confirmation that affinity-purified anti-CLIC4 polyclonal antibody also immunoprecipitated dynamin I (again with 20 μl of immunocomplex in each lane). The dynamin was detected with a goat anti-(dynamin I) antibody (Santa Cruz). (**b**) Samples (6, 12 and 20 μl) of the immunocomplex obtained after immunoprecipitation of β-actin, α-tubulin and 14-3-3ζ from 1 ml of cytosol with anti-CLIC4 antiserum at 1:100 dilution. The primary antibodies used for detection were mouse monoclonal anti-(β-actin) and anti-tubulin antibodies (Sigma) and a sheep antiserum raised against the unique EILNSPEKAC motif of 14-3-3ζ/β. Relative molecular masses (estimated from molecular mass markers) are indicated at the right.

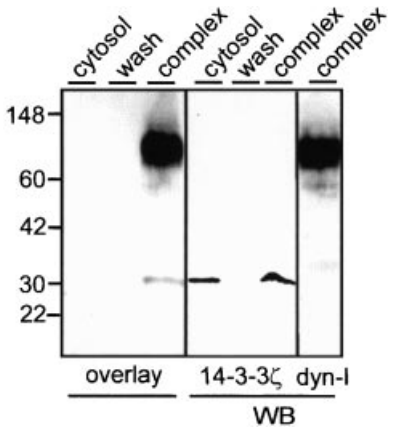

Figure 3 Gel-overlay assays confirm that CLIC4 binds directly to dynamin I and 14-3-3ζ

For the gel-overlay panel, rat brain cytosol (25 μg of protein), 20% of the final affinity column washings (see the text) and 20% of the salt-eluted, affinity-purified complex from 100 ml of cytoplasm (containing 500 mg of protein) were subjected to SDS/PAGE [10% (w/v) gel] and overlaid with CLIC4cd. After extensive washing, bound CLIC4 was revealed by anti-CLIC4 immunoblotting. Note that CLIC4 bound only to regions corresponding to two protein bands and did not bind to any proteins in the column washings. The Western blot (WB) panels contained identical samples probed with a rabbit anti-(14-3-3ζ) polyclonal antibody; the sample of affinity-purified complex in the final lane was probed with goat anti-(dynamin I) polyclonal antibody (dyn-I; Santa Cruz). The positions of molecular mass markers are indicated (in kDa) at the left.

down assays (e.g. Figure 5) with rabbit muscle α-actin and platelet β-actin and γ-actin. CLIC4 did not seem to interact directly with either α or β (or γ) F-actins, although cofilin bound readily to (β/γ)-actin at pH 6.5, as shown. This suggests that CLIC4 might bind to F-actin indirectly or that it might bind to non-polymerized G-actin.

Cellular distribution of CLIC4

Apart from the important roles of dynamin I in synaptic vesicle endocytosis in neurons, dynamin isoforms are known to be involved in receptor-mediated endocytosis, Golgi vesicle formation, phagocytosis, mitochondrial morphogenesis and remodelling of the actin cytoskeleton [31,32]. They are also closely involved in the mechanism of caveolar endocytosis [33,34]. We examined the possible interaction between CLIC4 and dynamin in the context of caveolar endocytosis in HEK-293 cells, given a previous report suggesting that human CLIC4 partly co-localizes with caveolae [22].

The primary sequence of human CLIC4 is almost identical with that of rat brain CLIC4 and it is expressed in HEK-293 cells, in which it cross-reacted with our anti-(rat CLIC4) antiserum (results not shown). However, the endogenous protein is often undetectable, suggesting that it might be expressed at relatively low levels. Western blotting of membrane fractions from HEK-293 cells stably transfected with CLIC4 (Figure 6a) confirmed the typical dual localization of (in this case mainly recombinant) protein in both membrane fractions and cytosol. Surprisingly, when we expressed dynamin I in control HEK-293 cells that were not stably transfected with CLIC4, we could then easily detect endogenous CLIC4, which appeared in the high-speed pellet (mixed mitochondrial/microsomal membrane) fraction (Figure 6b). The most likely explanation for this result is that the expression of exogenous dynamin I partly relocalizes endogenous human CLIC4 from the cytosolic fraction to cell membranes.

To determine whether CLIC4 and caveolin 1 associate in HEK-293 cells, which would be consistent with an involvement of CLIC4 in caveolar endocytosis, we isolated detergent-resistant caveolar membranes from cells stably transfected with CLIC4, by using sucrose density gradients [35]. Although CLIC4 was partly associated with detergent-resistant membrane vesicles (Figure 6c, fractions 4–6), most of the recombinant protein was present in the solubilized protein fractions (9–12) and in the Triton X-100-insoluble membrane pellet. This suggested that only a minor proportion of CLIC4 was associated with free caveolae (Figure 6c).

Co-localization with markers of caveolar endocytosis

We examined the association of CLIC4 with caveolin in HEK-293 cells by indirect immunofluorescence. Recombinant CLIC4 was partly co-localized with caveolin after fixation and co-staining (Figures 7a and 7b). To test the idea that CLIC4 is associated with functional caveolae, we preincubated CLIC4-transfected HEK-293 cells with FITC-labelled CT B subunit

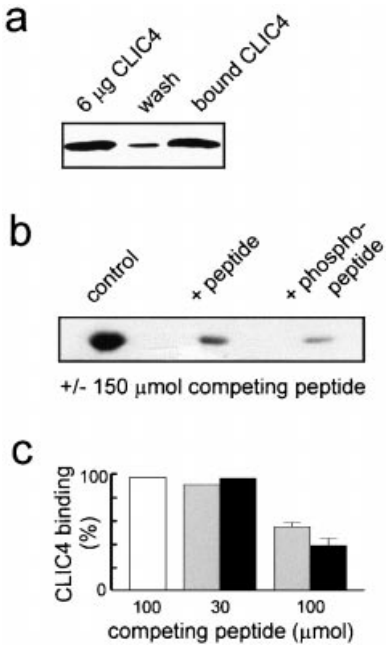

Figure 4 Reverse binding assay with immobilized 14-3-3ζ

14-3-3ζ was expressed as a GST fusion protein; 1.0 mg of 14-3-3ζ was coupled to glutathione beads and equilibrated with 300 μg of recombinant CLIC4cd. The beads were placed in a column and washed with 100 ml of binding buffer (see the Materials and methods section). (**a**) CLIC4 binds to 14-3-3ζ. The final 10 ml of wash buffer, and bound CLIC4 eluted by 2 M NaCl, were each collected and concentrated to 66 μl. The wash sample and the complex were each mixed with 33 μl of 3 × SDS sample buffer; 20 μl samples, now containing (in the case of the complex) 20% of the bound and released CLIC4cd, were subjected to SDS/PAGE [10% (w/v) gel] and immunoblotted with anti-CLIC4 antiserum followed by ECL detection. The first lane contained 6 μg of recombinant CLIC4cd. (**b**) CLIC4 binding to 14-3-3ζ is decreased in the presence of Raf peptides. The control lane shows CLIC4 bound to 14-3-3ζ in an aliquot of washed beads in the absence of competing peptides. The subsequent lanes contained the same amount of washed beads from experiments in which CLIC4 binding had been performed under exactly the same conditions, except for the presence of 150 μM non-phosphorylated and phosphorylated Raf peptides respectively (see the text). (**c**) CLIC4 binding to 14-3-3ζ was not decreased by 100 μmol of control peptide (white bar; see the text for details) or by 30 μmol of non-phosphorylated (grey bars) or phosphorylated (black bars) Raf peptides. Binding was decreased in the presence of 100 μmol Raf peptides; inhibition by the phosphopeptide was significantly enhanced ($P < 0.05$) in comparison with that by the non-phosphorylated peptide. Each bar is the mean value from two independent experiments or, where S.E.M. values are shown, three experiments (means were compared by Student's t test). In each experiment, densitometric comparisons were performed with non-inhibited binding analysed on the same gel.

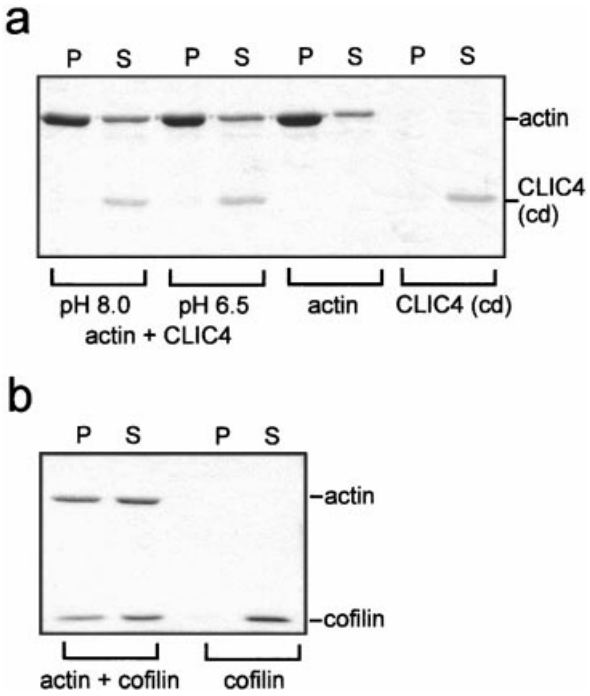

Figure 5 Actin spin-down assay

(**a**) CLIC4 does not bind to polymerized actin. G-actin (5 μM) was polymerized with or without 5 μM CLIC4cd at a pH of 8.0 or 6.5, as indicated, in the presence of 50 mM KCl, 0.2 mM ATP, 1 mM $MgCl_2$ and 0.2 mM $CaCl_2$. Equal volumes of supernatants (S) containing non-polymerized G-actin and pellets (P) containing F-actin were subjected to SDS/PAGE [10% (w/v) gel] and Coomassie staining. CLIC4cd was included on its own (at 5 μM) in parallel tubes (at pH 6.5). CLIC4cd remained in the supernatant fraction and did not co-sediment with F-actin. Also note that the ratio of G-actin to F-actin was unaltered in the presence of CLIC4. The same results were obtained with either α-actin or a mixture of β-actin and γ-actin (see the text). (**b**) Cofilin binds to actin under conditions similar to those in (**a**) (pH 6.5, positive control). Note the appearance of cofilin in the pellet fraction, with polymerized actin.

[33,34] before fixation (Figures 7c and 7d). CT B subunit was readily taken up by HEK-293 cells; cells that were also transfected with CLIC4 (detected with a TRITC-coupled secondary antibody) showed partial co-localization of CLIC4 with the toxin. Pixel-by-pixel reconstruction of a quadrant of the data in Figure 7(c), in a single Z-plane corresponding to a cell slice 1 μm thick, showed that the co-localization was limited to approx. 10% of the recombinant CLIC4 at this resolution.

In further experiments on cells transfected with CLIC4, we post-stained the actin cytoskeleton with TRITC-labelled phalloidin (after fixation and permeabilization), or co-stained transfected dynamin I. We noted only partial co-localization of CLIC4 with both proteins (results not shown). CLIC4 is widely distributed in both membrane and soluble compartments; it was not surprising that only a small proportion of the cellular CLIC4 was found to be associated with endogenous actin or recombinant dynamin I. We also exposed CLIC4-transfected cells to TRITC-labelled transferrin and removed surface-bound transferrin by washing with acid before fixation and CLIC4 counterstaining. This showed no clear evidence for co-localization of CLIC4 with the labelled, endocytosed transferrin (results not shown).

DISCUSSION

Identity of intracellular anion channels

Anion-selective channels have been reconstituted from secretory vesicles [9]; Cl^- channels might regulate the pH of some endosomal compartments [12]. The molecular identity of intracellular Cl^- channels has remained unclear, but CLIC proteins are currently among the leading candidates. The reconstitution of channel activity from pure CLIC1 is highly significant [27], but it is important to stress that the channels have not been shown to correspond to any ion channel normally present in cells. We therefore cannot yet be sure that the behaviour of CLIC proteins *in vitro* is relevant to their behaviour *in vivo*. p64 itself was first isolated by indanoyloxyacetic acid (IAA) affinity chromatography and identified as a putative anion channel protein because anti-p64 antibodies reduced IAA-sensitive Cl^- transport in a kidney microsomal membrane vesicle fraction [36]. However, although kidney microsomes had previously been demonstrated to contain Cl^- channels [37], the channels did not correspond to the anion channel activity recorded from p64-containing HeLa

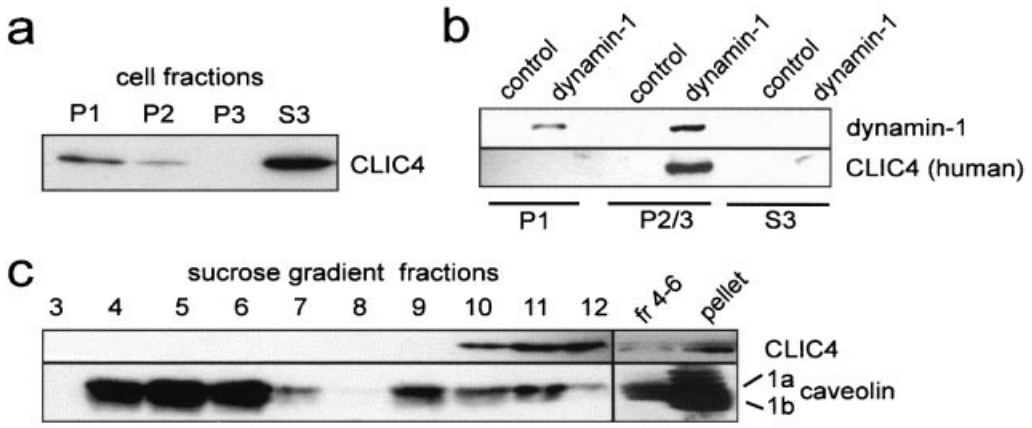

Figure 6 Intracellular localization of CLIC4

(**a**) Localization of recombinant CLIC4. HEK-293 cells stably transfected with CLIC4 were fractionated to give unbroken cells and nuclear membranes (P1, pellet from 5000 ***g*** for 10 min), a fraction enriched in mitochondrial membranes (P2, pellet from 10000 ***g*** for 20 min), a fraction containing microsomal membranes (P3, pellet from 300000 ***g*** for 20 min), and a supernatant (S3) containing soluble proteins. Protein (10 μg) from each fraction was subjected to SDS/PAGE [10% (w/v) gel]; CLIC4 was detected by Western blotting. (**b**) Recombinant dynamin I localizes endogenous human CLIC4 to membranes in HEK-293 cells. HEK-293 cells were fractionated as in (**a**) except that the P2 and P3 fractions were combined. In cells expressing recombinant dynamin I (upper panel), native human CLIC4 (which is undetectable in the control cells) appeared in the P2/3 membrane fraction. All lanes contained 20 μg of protein. (**c**) Partial co-localization of recombinant CLIC4 with caveolar membranes. HEK-293 monolayers (equivalent to 750 cm^2) were homogenized in a buffer containing 1% (v/v) Triton-X 100, mixed with a concentrated sucrose solution and placed at the bottom of an SW41 (Beckman) centrifuge tube to form a 2 ml 40% (w/v) sucrose step. This was overlaid with 6 ml of 30% (w/v) sucrose and 4 ml of 5% (w/v) sucrose and left to stand for 60 min. Twelve 1 ml fractions (numbered from top to bottom) were collected after centrifugation for 16 h at 175000 g_{av}. In another tube, the proteins in fractions 4–6 (corresponding to free caveolae) and the proteins in the resuspended detergent-insoluble pellet were precipitated by 30% (w/v) trichloroacetic acid and resuspended in 1 ml of lysis buffer. A 20 μl sample of each fraction was analysed by SDS/PAGE [12.5% (w/v) gel] and immunoblotting to detect CLIC4 and caveolin 1 (a and b isoforms).

cell membranes [38]. Unfortunately, the effect of IAA compounds was not reported in the latter study.

Proteins associated with CLIC4

Dynamin I is the neuron-specific isoform of dynamin [39], the mechanoenzyme essential for the fission of synaptic vesicles during endocytosis [40]. Although the association of CLIC4 with brain dynamin I is compatible with a significant involvement in synaptic vesicle endocytosis, CLIC4 is also widely expressed in other tissues, in which it might interact with one or more of the many spliced forms of dynamin II or dynamin III [31–33]. Caveolin 3 (and possibly also caveolin 1) has a WW-like domain [41], compatible with a WW-domain-binding motif (PPKY) in CLIC4 (Figure 8). Our findings in HEK-293 cells suggest that recombinant CLIC4 co-localizes partly with caveolin, as reported previously [22], and that recombinant CLIC4 is, in part, associated with caveolar endocytosis. However, CLIC4 has a widespread distribution in both membranes and cytosol; only a minor proportion of total cellular CLIC4 is localized to caveolar membranes.

Although neurons do not contain classical caveolae, they do contain typical caveolar glycolipids concentrated in specific regions of the synaptic membrane [42]. These regions probably correspond to plasma-membrane microdomains (micropatches) that contain caveolae-like proteins similar to those localized in putative neuronal growth cone micropatches in axonal lamellipodia and filopodia [43]. Dynamin I expression is known to be increased during neurite outgrowth [44]; conversely, reducing the expression of dynamin I decreases neurite formation [45]. We further note that CLIC4cd has a potential motif recognized by SH3-domain proteins (Figure 8) (compare with p64 [18]). SH3 domains are common in proteins associated with endocytosis; this region could represent an important, regulated targeting motif in CLIC4.

Our observation that the expression of dynamin I in HEK-293 cells drives endogenous CLIC4 to membranes, presumably at the expense of the large cytoplasmic protein pool, confirms an interaction *in vivo* between the two proteins, but the interpretation of this experiment is not straightforward. For example, the expression of a non-native isoform of dynamin might sequester binding partners for endogenous isoforms, specifically ubiquitously expressed dynamin II, and this could affect the distribution of CLIC4 indirectly. Increased expression of dynamin II has been shown to promote apoptosis [46], introducing the idea that the dynamins might function as signalling-linked GTPases as well as being mechanoenzymes. It would be of interest to determine whether the overexpression of dynamin I (or CLIC4) has any effect on apoptosis in cells, especially given the conjecture that the murine version of CLIC4 that associates with mitochondrial membranes (mtCLIC) can promote apoptosis [23]. Future identification of the interacting domains of CLIC4 and dynamin isoforms will provide further clues to cellular mechanisms and will suggest rational ways in which dominant-negative CLIC4 mutants can be constructed to investigate the cellular functions of CLIC4 in more detail.

The brain CLIC4 protein complex also contains α-tubulin. Interestingly, a previous immunohistochemical study [21] revealed a close association of CLIC4 with rat brain microtubules, in addition to partial co-localization with large dense core neurosecretory vesicles. Our results linking CLIC4 and tubulin at the protein level reflect the possibility that dynamin and tubulin might interact *in vivo*, which is consistent with the original characterization of brain dynamin as a microtubule-binding protein [39]. However, the relevance of the latter association remains to be determined; it might arise from the fortuitous occlusion of dynamin inside microtubules. Although the functional association of these two proteins *in vivo* remains controversial, tubulin does seem to be localized (via a lipid anchor) to caveolae-like domains in neuronal plasma membranes [47].

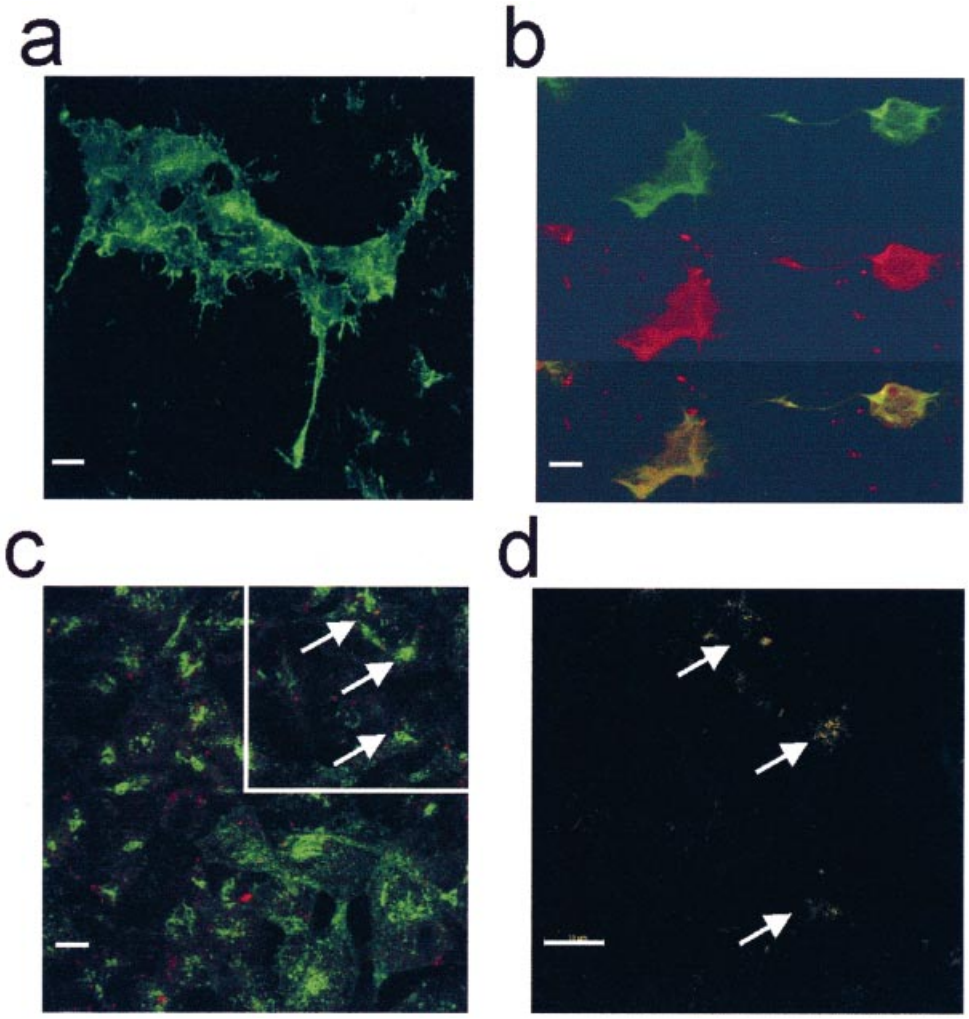

Figure 7 CLIC4 and caveolar endocytosis in HEK-293 cells

(**a**) FITC-stained CLIC4 24 h after transient transfection of a large group of HEK-293 cells, showing generalized cytoplasmic distribution and some localization to membranes in or near the plasmalemma. Endogenous CLIC4 and preimmune serum produced no detectable signals (results not shown). (**b**) FITC-stained CLIC4 24 h after transient transfection of HEK-293 cells (top panel), immunofluorescence staining for caveolin 1 labelled with a TRITC-coupled secondary antibody (middle panel) and overlay to show partial (yellow) co-localization (bottom panel). (**c**) At 40 min before washing and fixation, FITC-labelled CT B subunit was added to HEK-293 cells containing relatively low levels of recombinant CLIC4 (stained with a TRITC-coupled secondary antibody). The upper right quadrant (boxed) with three regions of concentrated CT B chain localization (arrowed) was analysed in more detail (shown in **d**). (**d**) Pixel-by-pixel reconstruction and overlay of a single slab 1 μm thick taken from the upper right quadrant (white box) of the cell region in (**c**). Yellow dots show co-localization of TRITC and FITC staining (8% of the TRITC-labelled CLIC4 was co-localized with FITC-labelled CT B subunit). Scale bars, 10 μm.

This is consistent with the partial localization of CLIC4 to caveolae.

14-3-3 proteins bound directly or indirectly in the CLIC4 protein complex could have one of several different roles, including the mediation of protein-kinase-C-dependent CLIC4 phosphorylation [17]. Alternatively, they might function as adapter proteins, dimerizing with other 14-3-3 subunits coupled to other proteins or signalling complexes [48]. Certain 14-3-3 isoforms, including 14-3-3ζ and 14-3-3ϵ, are co-localized in synaptosomes and synaptic membranes [49]. CLIC4 might contain a variation of a recognized 14-3-3-binding motif (see Figure 8); further exploration of these possible interactions could be revealing. Raf peptides containing a 14-3-3 protein-binding motif decrease the binding of CLIC4 to 14-3-3, especially when the Raf peptide is phosphorylated (Figures 4b and 4c). This reinforces the idea that the binding of CLIC4 to 14-3-3 proteins might be physiologically regulated in the cell. The presence of creatine kinase in the CLIC4 protein complex is intriguing but unexplained, although creatine kinase has previously been observed to co-purify with 14-3-3 proteins (A. Aitken and Y. Soneji, unpublished work).

Like CLIC4, CLIC5 also associates (directly or indirectly) with actin, in apical placental trophoblast [26]. In PC12 cells, a rat adrenal medullary phaeochromocytoma cell line that shows regulated secretion, neurosecretory granule movement near the cell membrane is strongly influenced by the actin cytoskeleton in multiple ways, as shown very elegantly in a recent total-internal-reflection fluorescence microsocopy study [50]. The actin cytoskeleton must also be intact for normal receptor-mediated endocytosis [51]; dynamin is known to interact with the actin-binding protein profilin [29]. The poorly understood connection between actin and endocytosis is further reinforced by the potential physical link that we have discovered between actin and dynamin mediated by CLIC4. Dynamin II also binds to the SH3

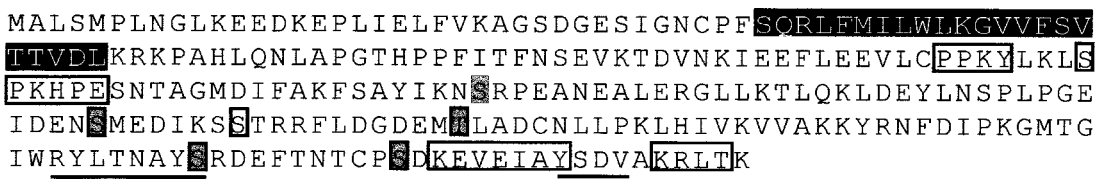

Figure 8 Potential protein interaction motifs in CLIC4

Complete amino acid sequence of rat brain CLIC4 [17], showing the predicted transmembrane domain (highlighted), boxed motifs (from the N-terminus to the C-terminus) that potentially bind to WW-domain proteins (PPKY) or SH3-domain proteins (PKHPE), as well as consensus tyrosine kinase (KEVEIAY) and cAMP-dependent (KRLT) phosphorylation sites. Serine (and threonine) residues in consensus protein-kinase-C-dependent or casein-kinase-2-dependent phosphorylation sites are shown boxed and/or with a grey background respectively. The underlined RYLTNAYS motif is similar to the $RX_{1,2}SX_{2,3}S$ motif of 14-3-3-binding proteins; phosphorylation of the tyrosine residue in the underlined YSDV motif (following the consensus tyrosine kinase phosphorylation site) could potentially contribute a motif recognized by SH2-domain proteins.

domain protein syndapin 1 and to cortactin, an actin-bundling protein in membrane ruffles that is now known to be functionally involved in the control of cell shape [52].

Possible cellular roles for CLIC4

Could the 'anion channel protein' CLIC4 be recruited to micropatches involved in the control of cell shape? This recalls suggestions by Okada [53] on cell and organelle volume control, a largely unresolved area of major importance in biology. At least some of the net efflux of anions during the regulatory volume decrease after osmotic cell swelling is through anion channels but their identity remains controversial. Okada has proposed [14] that cytoskeletal rearrangements during cell swelling might physically redistribute volume-regulated anion channels, for example moving them from membrane infoldings to the exposed plasma membrane. The association of previously separate proteins that might then be able to activate ion channels could also be important. Consistent with this novel 'occluded anion channel' hypothesis are the observations that caveolins modulate volume-regulated anion channel activity [54] and that caveolae cluster centrally in cells exposed to hyperosmotic medium, provided that the actin and microtubule cytoskeletons both remain intact [55].

Drawing on this idea, we speculate that CLIC4 (and other CLIC proteins) might be associated with anion channel activity in cells, in part because they are involved in the redistribution of channel-containing vesicles during vesicle trafficking, including membrane redistribution and retrieval associated with actin-mediated changes in cell shape [31,51]. Because of the association of CLIC4 with dynamin, this function might be performed near sites of vesicle fission. It is noteworthy that the multiple cellular sites described for CLIC4 (including secretory vesicles, mitochondrial membranes and caveolae) all correspond to sites in the cell where dynamin or dynamin-like proteins have been implicated in vesicle trafficking or the control of organelle (especially mitochondrion) shape [31,32]. An attractive unifying hypothesis that might also explain why purified CLIC1 exhibits ion channel activity [27] is that CLIC proteins are membrane-active. The membrane-attaching PH domain of dynamin is itself known to be membrane-active; this might further help to destabilize membranes. The physical association of dynamin with another membrane-active protein, CLIC4, could be particularly relevant to membrane fission; we are currently exploring these possibilities.

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