ERK7, a member of the mitogen-activated protein kinase family, has a carboxyl-terminal tail that is required for ERK7 activation, cellular localization, and its ability to inhibit DNA synthesis. To identify proteins that interact with ERK7, we utilized a yeast two-hybrid screen with the COOH-terminal tail of ERK7 as bait and isolated the cDNA for a novel protein termed CLIC3. The interaction between CLIC3 and ERK7 in mammalian cells was confirmed by co-immunoprecipitation. CLIC3 has significant homology to human intracellular chloride channels 1 (NCC27/CLIC1) and 2 and bovine kidney chloride channel p64. Like NCC27/CLIC1, CLIC3 is predominantly localized in the nucleus and stimulates chloride conductance when expressed in cells. Taken together, these results suggest that CLIC3 is a new member of the human CLIC family. The observed interaction between CLIC3 and ERK7 is the first demonstration of a stable complex between a protein that activates chloride ion transport and a member of the mitogen-activated protein kinase family of signal transducers. The specific association of CLIC3 with the COOH-terminal tail of ERK7 suggests that CLIC3 may play a role in the regulation of cell growth.

Chloride channels are a diverse group of proteins that regulate fundamental cellular processes including stabilization of cell membrane potential (1, 2), transepithelial transport, maintenance of intracellular pH (3), and regulation of cell volume (4, 5). Various chloride channels have also been implicated in human hereditary diseases such as the dominant and recessive forms of myotonia (6), X-linked hereditary nephrolithiasis (7), and cystic fibrosis (8). Although the majority of chloride channels characterized to date have been localized to the plasma membrane (9), a new family of proteins that activate intracellular chloride permeability has been identified within the past few years.

Termed the "chloride intracellular channel" or CLIC1 family (10), at least two members have been described. The CLIC family has homology to p64, a bovine kidney microsomal chloride ion transporter characterized by Landry and colleagues (11–13). A rat homologue of p64 (p64G1) has also been cloned and shown to play a role in intracellular chloride ion transport within the endoplasmic reticulum (14). The highly related but distinct CLIC class of chloride ion transporters shares homology with the COOH-terminal half of p64 (10, 15). The prototype for this family is NCC27/CLIC1 (15), a small protein of 241 amino acids that is expressed primarily in the nucleus and exhibits both nuclear and plasma membrane chloride ion channel activity. CLIC2, a 243-amino acid protein that shares a putative nuclear localization signal, has not yet been characterized but has been mapped to a region of the chromosome that codes for proteins important in a number of diseases. Although the specific function of these proteins is not yet known, their high degree of conservation and unusual cellular localization suggest that they play a central role in cellular function. Since the CLIC proteins lack the membrane-spanning domains characteristic of channel proteins and are largely intracellular, it is possible that they are not themselves chloride channels but instead function as activators of chloride channels.

There is increasing evidence indicating that cellular signaling cascades play a fundamental role as regulators of ion channels. For example, the high voltage-activated Ca\(^{2+}\) channel and Ca\(^{2+}\)-activated K\(^+\) channels can be modulated by growth factors (16, 17). Both NMDA receptors and the human Kv1.5 K\(^+\) channel were shown to be regulated by the tyrosine kinase Src (18, 19). In lymphocytes, the tyrosine kinase p56\(^{lck}\) mediates activation of a swelling-activated chloride channel (20) and also activates an outwardly rectifying chloride channel during Fas-mediated apoptosis (21). However, little is known about how signaling pathways modulate chloride channels.

One of the major signaling cascades activated by growth and differentiating factors in cells is the mitogen-activated protein (MAP) kinase cascade. A superfamily of highly homologous proline-directed serine/threonine kinases, MAP kinases are regulated through distinct signaling cascades involving upstream protein kinases that phosphorylate both tyrosine and threonine residues in a Thr-X-Tyr (TXY) motif positioned within the activation loop of MAP kinases (reviewed in Ref. 22). Recently, a novel 60-kDa member of the MAP kinase family, termed extracellular signal-regulated kinase 7 (ERK7), has been cloned and characterized (23). Although it has the signature TEF activation motif of ERK1 and ERK2, ERK7 is significantly different from previously identified ERKs. First, ERK7 does not appear to be activated either by extracellular stimuli that typically activate ERK1 or ERK2 or by common activators of the JNK or p38 pathways. Instead, this novel MAP kinase has appreciable constitutive activity in serum-starved cells, and this activity requires the presence of a COOH-terminal...
The expression is driven in the nucleus in a COOH-terminal tail-dependent fashion. Finally, ERK7 can function as a negative regulator of cell growth, and this activity is dependent on the presence of the COOH-terminal tail but independent of its kinase activity. Taken together, these results describe a new type of MAP kinase family member whereby interactions via its COOH-terminal tail, rather than extracellular signal-mediated activation cascades, regulate its activity, its localization, and its function.

In order to elucidate the mechanism by which ERK7 is regulated, we identified proteins that interact with the COOH-terminal tail of ERK7 using a two-hybrid screen. In the present study, we describe the isolation and characterization of a new member of the CLIC family of intracellular chloride channels termed CLIC3 that specifically associates with ERK7. Like NCC27/CLIC1, CLIC3 is a small protein that is localized primarily in the nucleus and stimulates chloride ion channel activity. The observed association of CLIC3 with a member of the MAP kinase family, in conjunction with the nuclear localization of both of these proteins, raises the possibility that CLIC3 may participate in cellular growth control.

Materials and Methods
Plasmids and Antibodies—The plasmids were the same as previously described. The antibodies used were mouse anti-FLAG M5 and rabbit anti-HA antibody. Enhanced Chemiluminescence Reagents (Amersham Pharmacia Biotech) were purchased from NEN Life Science Products. The pCMV-CLIC3 expression vector was a gift from V. Sukhatme. Plasmid DNAs were sequenced by the Interdisciplinary Center for Biotechnology Research DNA sequencing core laboratory at the University of Florida or by the University of Chicago Cancer Research Center DNA sequencing facility.

Yeast Two-hybrid Screen—The BamHI-NcoI fragment of ERK7 containing the COOH-terminal tail (25) was subcloned into a Bluescript II KS (+) vector to construct pB-ERK7. The EcoRI-NcoI fragment from pB-ERK7 was fused into a LexA DNA binding domain in pEG202, resulting in the pEG202-ERK7 construct. The human fetal brain library, kindly provided by Dr. Roger Brent, was made from a 22-week-old human fetal frontal cortex and was used to search for novel proteins that interact with ERK7. In a screen of ~1 × 10^6 primary transformants, two clones (S9 and S46) showing strong interaction with ERK7 were obtained. Yeast transformation and routine yeast work were performed as described (25).

Tissue Northern Blot—The EcoRI fragment from pCLIC3 plasmid was isolated and radiolabeled with 32P using the Megaprime DNA labeling system (Amersham Pharmacia Biotech) and hybridized to a poly(A)- RNA human tissue Northern blot (CLONTECH). Hybridization was performed according to the user manual (CLONTECH).

Epitope Tagging—Primer A (5′-AGACTCAAAAGGGAGGATGAGAACGTCCTGCTCATTACGCTACCGCAGG-3′) and primer B (5′-GCTGAGAACAGATACCTTTGATTTGG-3′) were used to clone the CLIC3 from S9 cDNA by PCR with ID Proof Taq (ID Lab). The PCR products were ligated into the pCR3.1 TA vector (Invitrogen) to construct pCLIC3. Primer A included nucleotides coding for the FLAG epitope right after the ATG codon and the 23 nucleotides missing in the 5′ end of S9 cDNA. The EcoRI fragment containing CLIC3 was subcloned into a pGEX-2T vector to express the GST-CLIC3 fusion protein in bacterial BL21 cells. GST fusion proteins were prepared using a GST purification system (Amersham Pharmacia Biotech). An HA tag, YFP/DVDYPI, was inserted right after the initiating methionine of ERK7 by PCR (25). The HA-K40R ERK7 mutant was generated by unique site elimination mutagenesis (26).

Cell Culture and Transient Transfections—COS, CV-1, and LTK cells were grown in Dulbecco’s modified Eagle’s medium supplemented with antibiotics (50 units/ml penicillin and 50 μg/ml streptomycin) and 10% fetal bovine serum in a 95% air, 5% CO2 incubator at 37 °C. COS and CV-1 cells were transfected with a total of 10 μg of plasmid DNA, and transfection was performed with the TransIT™ polyamine transfection reagent according to the manufacturer’s instructions (Panvera, Madison, WI). The LipofectAMINE™ Reagent (Life Technologies, Inc.) was used for transfection of LTK cells. The pGreen Lantern-1 (Life Technologies, Inc.) green fluorescent protein (GFP) or the pCMV-β-galactosa- siade expression vectors were co-transfected with other plasmids as indicated in the text for visualizing transfected cells or as indicators of transfection efficiency.

Cell Lysis and Western Blot—Cultured cells were washed twice with ice-cold phosphate-buffered saline and lysed in 1% Triton-X-100 buffer containing 50 mM Tris-HCl, pH 7.5, 10 mM NaCl, 50 mM NaF, 1% Triton X-100, 40 mM β-glycerophosphate, 2 mM EDTA, 1 mM sodium orthovanadate, 20 μM phenylmethylsulfonyl fluoride, 1 μg/ml aprotonin, 1 μg/ml leupeptin, and 20 mM p-nitrophenyl phosphate. Cell extracts were separated by 10% or 12% SDS-polyacrylamide gels. Proteins were transferred to a nitrocellulose membrane. Western blot analysis was performed as described previously (27).

Immunoprecipitation and in Vitro Kinase Assays—Immunoprecipitation was performed as described previously (28). In order to assay ERK7 kinase activity, COS cells were transfected with HA-ERK7 or HA-ERK7-K43R. After 24–48 h, cells were lysed in 0.5% Triton X-100 buffer, and ERK7 and ERK7-K43R were immunoprecipitated using the anti-HA antibody. The immunocomplex was washed three times with lysine buffer and twice with kinase buffer (20 mM Hepes, pH 7.4, 10 mM MgCl2, 1 mM DTT, 0.2 mM sodium vanadate, 10 mM p-nitrophenyl phosphate). 2 μg of each substrate were used per reaction in kinase buffer containing 5 μCi of [γ-32P]ATP. The kinase reaction was incubated at 30 °C for 30 min and terminated by boiling the mixtures in sample buffer for 5 min. The reaction products were separated by 8% SDS-PAGE.

Immunocytochemistry—The CV-1 cells were plated on coverslips and incubated overnight. Cells were transfected with FLAG-CLIC3 and pCMV-β-galactosidase or HA-ERK7 using the TransIT-LT1 reagent. After 48 h, the cells were fixed in 10% formaldehyde solution (Fisher) for 15 min, washed with phosphate-buffered saline, blocked, and incubated simultaneously with mouse anti-FLAG M4, and rabbit polyclonal anti-β-galactosidase antibody (5 Prime→ 3 Prime, Boulder, CO) or rat high affinity anti-HA antibody. After 1 h of incubation at room temperature, the cells were washed and then incubated with Texas Red-conjugated anti-mouse and fluorescein isothiocyanate-conjugated anti-rabbit antibodies or fluorescein isothiocyanate-conjugated anti-rat antibody (Molecular Probes, Inc., Eugene, OR). The stained cells were analyzed using a Zeiss Axioplan fluorescent microscope.

Electrophysiological Experiments—LTK cells were transfected with both FLAG-CLIC3 and GFP or with GFP alone. Electrophysiological experiments were performed as described (10). The affected DNA was excised from an M13 plasmid and sequenced by the DNA sequencing core laboratory at the University of Florida or by the Interdisciplinary Center for Biotechnology Research DNA sequencing core laboratory at the University of Florida or by the University of Chicago Cancer Research Center DNA sequencing facility. Yeast cells were identified by GFP fluorescence. Membrane voltage was controlled with a voltage clamp in the whole-cell, tight-seal configuration (29). The resistance was 2–3 MΩ. Patch pipettes contained (in mM): KCl, 135; CaCl2, 1; MgCl2, 1; K2EGTA, 10; HEPES, 10; adjusted to pH 7.2 by the addition of KOH. The standard extracellular saline contained (in mM): NaCl, 140; KCl, 2.5; CaCl2, 3; MgCl2, 7; glucose, 15; HEPES, 10; adjusted to pH 7.4 by the addition of NaOH. A low chloride saline was made by substituting NaCl with sodium gluconate. Membrane potential was normally held at −70 mV. A current-voltage relation was measured by recording the current while the voltage was changed between −100 and +80 mV at a constant rate of 1.4 V/s. No correction was made for the series resistance of the patch pipette. Junction potentials were corrected as described in Ref. 30. Currents were low-pass filtered (8-pole with 3 dB attenuation at 3 kHz), digitized at 2 KHz, and subsequently analyzed with custom software written in Axobasic (Axon Instruments Inc.).

Results
Isolation of the Clone Encoding CLIC3, a Protein That Interacts with the COOH-terminal Tail of ERK7—In order to elucidate the mechanism by which the COOH-terminal tail of ERK7 regulates ERK7 activity and function, we screened for interacting proteins using the LexA-based yeast two-hybrid system (25). A restriction fragment of the ERK7 cDNA containing the COOH-terminal tail was used as bait for screening a human fetal brain library. Two cDNA clones (S9 and S46) out of 1 × 10^6 transformants interacted strongly with the tail of ERK7 but not with other unrelated control proteins (data not shown).

Sequence analysis indicated that both of these clones contained the same partial-length cDNA and were missing 23 nucleotides at the 5′ end of the full-length cDNA. Searching the

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human EST Data Base revealed an overlapping cDNA clone (gb/N41765) that was missing the 3' end but had the complete 5' end of the gene. Together, the cDNA sequences from S9 and the EST clones encoded a polypeptide of 208 amino acids with a predicted Mr of 23,569. The potential initiating methionine codon is right after a stop codon in the same reading frame and occurs in a consensus region favorable for initiation of translation in higher eukaryotes (31). Fig. 1A shows the nucleotide and predicted amino acid sequences of the protein encoded by the S9 clone. Comparison of the deduced amino acid sequence to other sequences in the Swiss Protein Data Base revealed a significant homology to human chloride intracellular channel 1 (NCC27/CLIC1) (15) and chloride intracellular channel 2 (CLIC2) (10). The protein encoded by S9 had 48–49% identity and 60–61% similarity to NCC27/CLIC1 and CLIC2. Furthermore, the S9 protein shared 47% identity and 60% similarity with the COOH-terminal half of the bovine chloride channel p64 (11–13). Due to the high homology among these proteins, the protein encoded by the S9 clone was termed CLIC3 for chloride intracellular channel 3.

The multiple alignment of CLIC3 with NCC27/CLIC1 and CLIC2 is shown in Fig. 1B. A strongly hydrophobic region in CLIC3, from amino acids 137–155 (Fig. 1A), was found by Kyte-Doolittle hydrophobicity analysis (32). This region represents a potential transmembrane domain that is very conserved among the CLIC genes. Searching for other possible motifs in CLIC3 revealed two potential casein kinase II phosphorylation sites at Thr-13 and Thr-141, two potential protein kinase C phosphorylation sites at Thr-17 and Ser-130, and one potential N-myristoylation site at Gly-7.

In Vivo Interaction between CLIC3 and ERK7—We generated a full-length clone of CLIC3 by PCR extension of the S9 sequence and then determined whether the protein could be expressed in mammalian cells. The full-length CLIC3 cDNA was tagged with a sequence encoding the FLAG epitope at the 5' end, and then transiently transfected into COS cells. The transfected cell lysates were resolved by SDS-PAGE and immunoblotted with anti-FLAG antibody. A protein of 24 kDa, consistent with the predicted molecular mass of CLIC3, was recognized specifically by the anti-FLAG antibody in the cells transfected with CLIC3, but not in the cells transfected with the control vector (Fig. 2). To determine whether ERK7 and...
CLIC3 associate in vivo, COS cells were transiently co-transfected with either an expression vector for FLAG-CLIC3 or control vector. CLIC3 protein was detected in cell lysates by immunoblotting with an anti-FLAG antibody. B, co-immunoprecipitation of CLIC3 and ERK7 in COS cells. Cells were transiently transfected with expression vectors for either FLAG-CLIC3 (lane 1), HA-ERK7 (lane 2), or both FLAG-CLIC3 and HA-ERK7 (lane 3). Total cell lysates were either directly resolved by SDS-PAGE (lane 1) or first immunoprecipitated with anti-HA antibody and then resolved by SDS-PAGE (lanes 2 and 3). All samples were then immunoblotted with anti-FLAG antibody or anti-HA antibody. The upper blot was probed with anti-HA antibody, which detects HA-ERK7, and the lower blot was probed with anti-FLAG antibody, which detects FLAG-CLIC3.

CLIC3 associate in vivo, COS cells were transiently co-transfected with HA-tagged ERK7 and either FLAG-tagged CLIC3 or the control vector. After immunoprecipitation of the cell lysates with anti-HA antibody, the immunoprecipitates were resolved by SDS-PAGE and probed for expression of both the HA-ERK7 and FLAG-CLIC3 proteins by immunoblotting with anti-HA antibody and anti-FLAG antibody, respectively. As shown in Fig. 2B, the FLAG-CLIC3 protein co-precipitated with ERK7, indicating that these two proteins do associate in vivo. Surprisingly, when FLAG-CLIC3 was immunoprecipitated first from cells transfected with both CLIC3 and ERK7, no ERK7 was detected in the immunoprecipitates (data not shown). This result may be due to steric hindrance by the anti-FLAG antibody bound to protein A beads preventing interaction with ERK7.

**Tissue Distribution of CLIC3**—A Northern blot from CLONTECH containing purified poly(A)<sup>+</sup> RNA from different human tissues was used to analyze the mRNA expression of CLIC3. As shown in Fig. 3, CLIC3 is expressed as a single transcript of approximately 1.15 kilobase in several human tissues, indicating that CLIC3 is widely expressed. However, the level of CLIC3 expression varied, depending upon the particular tissue type. CLIC3 has a very high expression level in placenta and is abundantly expressed in lung and heart, suggesting that it may play an important role in these tissues. CLIC3 is also expressed at a much lower level in skeletal muscle, kidney, and pancreas, and could not be detected in brain.

**Localization of CLIC3**—If CLIC3 associates with ERK7 in vivo, it should co-localize with ERK7 in the cell. Since the intracellular location of ERK7 is predominantly in the cell nucleus (23), we determined whether CLIC3 is also localized in the nucleus. CV-1 cells were transfected with FLAG-CLIC3 as well as CMV-β-galactosidase to monitor transfected cells. After fixation, the cells were immunostained with anti-FLAG and anti-β-galactosidase antibodies as described under “Materials and Methods.” As shown in Fig. 4, the nuclei in the CLIC3-transfected cells were intensely stained with the anti-FLAG antibody, but weak staining was also observed in the cytoplasm. In contrast, the staining by the anti-β-galactosidase antibody was more evenly distributed between the nucleus and the cytoplasm. These results indicate that the FLAG-CLIC3 is located predominantly in the nucleus. Cells were also co-transfected with plasmids for both FLAG-CLIC3 and HA-ERK7 to compare the localization of both proteins directly. As shown in Fig. 4B, HA-ERK7 and CLIC3 co-localized principally in the nucleus. However, there is significantly more cytoplasmic expression of CLIC3 than ERK7, suggesting that CLIC3 may also function independently of ERK7. The nuclear localization of CLIC3 was confirmed using LTK cells (data not shown).

**CLIC3 Is Not a Direct Substrate of ERK7**—To determine whether CLIC3 could potentially be a direct substrate of ERK7, we performed an in vitro kinase assay using a GST-CLIC3 fusion protein as a substrate. A kinase-inactive mutant of ERK7, ERK7-K43R, was used as a negative control (23). Cell lysates extracted from COS cells transfected with either HA-ERK7 or HA-ERK7-K43R were immunoprecipitated and then incubated with either GST-CLIC3 or GST-Fos in kinase assay
buffer. In contrast to GST-Fos, no significant phosphorylation of CLIC3 by ERK7 was observed (Fig. 5). Although we cannot rule out the possibility that a phosphorylation site on CLIC3 was unavailable due to the addition of the GST, these results suggest that CLIC3 associates with, but is not a direct substrate of, ERK7.

**Electrophysiological Properties of CLIC3 in LTK Cells**

When FLAG-CLIC3 is overexpressed in LTK cells, some of the protein is expressed in the plasma membrane as well as the cytoplasm. A similar protein distribution was observed upon overexpression of NCC27/CLIC1 (15). Therefore, we measured the current-voltage properties of CLIC3 expressed in the plasma membrane of LTK cells, which have a very low background of chloride channel activity. We first measured the current-voltage properties of untransfected LTK cells or cells transfected with GFP alone using the voltage clamp technique in the whole cell configuration. As illustrated in Fig. 6A, the current-voltage relation measured in the normal extracellular saline solution was nearly linear between −100 and +80 mV with a slope of 5 GΩ (Fig. 6A, traces 1 and 3). A similar relation was observed when cells were superfused with a saline solution containing a low Cl− concentration (trace 2). In contrast, cells co-transfected with GFP and CLIC3 had two different behaviors. A minority of the cells behaved exactly like cells transfected with GFP alone; these cells had an input resistance of 4.3 ± 4.8 GΩ (range: 0.630–13.6 GΩ; n = 7) and a linear current-voltage relation, which was not changed by superfusing with a saline solution containing a low Cl− concentration. These cells probably express GFP but not CLIC3 at the plasma membrane. A majority of the cells exhibited an altered behavior; the input resistance of 646 ± 242 MΩ (range: 140–1169 MΩ; n = 8) was lower, and exposure to a saline solution containing a low Cl− concentration altered the current-voltage relation (Fig. 6B). Reducing the extracellular Cl− concentration from 152.5 mM to 22.5 mM reduced the outward current observed at positive potentials. These results indicate that CLIC3 mediates chloride ion transport across the membrane.

**DISCUSSION**

In an effort to identify proteins that associate with the COOH-terminal tail of ERK7, we isolated the intracellular chloride channel 3 (CLIC3) cDNA by a yeast two-hybrid screen. The interaction between CLIC3 and ERK7 was confirmed by co-immunoprecipitation studies in COS cells. CLIC3 is a novel protein that is highly related to human CLIC1 (NCC27/CLIC1), CLIC2, and bovine kidney chloride channel p64. A strongly hydrophobic region representing a transmembrane domain is very conserved among NCC27/CLIC1, CLIC2, and CLIC3. Like NCC27/CLIC1, CLIC3 localized predominantly in the nucleus and induced chloride conductance when expressed in cells. Overall, these results implicate CLIC3 as a new member of the human CLIC family.

To date, only one of the CLIC family members has been shown to have nuclear chloride ion transport activity. NCC27/CLIC1 can promote chloride transport across both the nuclear and plasma membranes (15). By analogy based upon sequence homology, cellular localization, and plasma membrane channel activity, it is likely that CLIC3 also has nuclear as well as plasma membrane ion transport activity. Since it is very difficult to detect nuclear ion channel activity, we focused on demonstrating the function of CLIC3 as an inducer of ion transport by probing the plasma membrane in transfected cells. Furthermore, the electrophysiological properties of NCC27/CLIC1 in the nuclear versus the plasma membranes were similar. The role of nuclear ion transport is not clear at the present time, but

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**Fig. 4.** Cellular localization of CLIC3 and ERK7. A, CV-1 cells were co-transfected with expression vectors for β-galactosidase and FLAG-tagged CLIC3. β-Galactosidase and CLIC3 were visualized by immunocytochemistry with anti-β-galactosidase (left panel) or anti-FLAG (middle panel) antibodies, respectively. The phase micrograph is shown in the right panel. B, CV-1 cells were co-transfected with expression vectors for FLAG-CLIC3 and HA-ERK7. CLIC3 and ERK7 were visualized by immunocytochemistry with anti-FLAG (left panel) or anti-HA (right panel) antibodies, respectively.

**Fig. 5.** Phosphorylation of GST-Fos but not GST-CLIC3 by ERK7. COS cells were transfected with an expression vector for either HA-ERK7 or HA-K43R (the kinase-deficient mutant). GST-CLIC3 or GST-Fos were isolated from bacteria and incubated with HA-ERK7 or HA-K43R immunoprecipitated from the transfected COS cells. Kinase buffer with [32P]ATP was added, and the samples were assayed for kinase activity as described under "Materials and Methods." Following incubation, the samples were resolved by SDS-PAGE and analyzed by autoradiography. The positions of the 32P-labeled GST-Fos and GST-CLIC3 bands are indicated.
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The existence of three discrete family members suggests that the role is an important one.

Based on the current-voltage relationship of the LTK cells transfected with CLIC3, we conclude that CLIC3 stimulates a chloride/anion ion conductance. For example, reducing the extracellular chloride concentration from 152.5 mM to 22.5 mM reduced the outward current observed at positive potentials. The change is easily explained if cells expressing the CLIC3 protein, unlike cells transfected with GFP only, have a relatively large membrane conductance to Cl\(^{-}\). When Cl\(^{-}\) is removed from the extracellular saline solution, fewer anions are available to enter resulting in a reduction of outward current. Since intracellular Cl\(^{-}\) ions are still present and able to permeate, the current-voltage relation observed at negative potentials is unchanged. Each curve crosses the voltage axes at a different point. In this case the shift in crossing point is +20 mV. Because this is less than the 48 mV predicted by the Nernst equation for a perfect Cl\(^{-}\) channel, the membrane must also be permeable to other ions. For example, gluconate used to replace Cl\(^{-}\) may be able to carry current with reduced efficiency. The Goldman-Hodgkin-Katz equation predicts a gluconate:Cl\(^{-}\) permeability ratio of 0.173. Based on this ratio, the pore formed by CLIC3 appears to be relatively large.

The CLIC family members are much smaller in length than the characterized chloride ion channels and lack the characteristic transmembrane chloride channel (10). Thus, CLIC3, which has a limited hydrophilic domain, must either multimerize or associate with other subunits if it indeed is part of a membrane channel. Despite the electrophysiological experimental results supporting the hypothesis that CLIC3 is a component of a Cl\(^{-}\) channel, it is also possible that CLIC3 may act as a regulator of a channel. One example of such a protein is pICln, which was cloned and initially identified as a swelling-induced chloride channel (33). Subsequently, Krapivinsky and co-workers (34) showed that anti-pICln monoclonal antibodies can block the native swelling-induced chloride channel in Xenopus oocytes, and that pICln was abundant in the cytoplasm and bound to actin. pICln was also shown to bind to a human homolog of yeast SKb1 protein (IBP72), which is associated with Shk1 kinase (35). Taken together, these results suggest that pICln may play an indirect role in Cl\(^{-}\) channel conductance, possibly via interactions with the cytoskeletal network. CLIC3, which is also associated with a kinase, may function in a similar manner.

Although CLIC3 is principally localized in the nucleus, no nuclear localization sequence was found. A conserved KKYR has been proposed as a nuclear localization signal motif in the 3’-ends of NCC27/CLIC1, CLIC2, and p64 (15), but this motif is not present in CLIC3. Therefore, it is possible that CLIC3 is transported into the nucleus via association with other proteins that are translocated to the nucleus. The observation that CLIC3 is not completely nuclear is consistent with the possibility. Since CLIC3 co-localizes with ERK7 in the nucleus, one potential role of ERK7 could be to bind CLIC3 and transport it to the nucleus.

ERK7, the MAP kinase family member that associates with CLIC3, is the newest member of the MAP kinase family. Among the extracellular signal-regulated or ERK family of MAP kinases, only four out of the seven enzymes designated ERKs have the signature TEY activation motif. These are ERKs 1, 2, 5, and 7. ERKs 1 and 2, the enzymes that were originally designated as MAP kinases, are a discrete size between 42 and 44 kDa. In contrast, ERKs 5 and 7 are larger due to the presence of a carboxyl-terminal tail that modulates the properties of the enzyme. Although ERK7 has the signature TEY activation motif of ERK1 and ERK2, this enzyme appears to be significantly different from other ERK family members (23). In contrast to previously reported ERKs, ERK7 has significant constitutive activity in serum-starved cells, is targeted to the nucleus in both active and inactive states, and can function as a negative regulator of growth independent of its kinase activity. In all cases, these properties are dependent upon the presence of the COOH-terminal tail. Thus, the interaction of ERK7 with CLIC3 may be very important in the action of the enzyme.

The functional consequences of the interaction between CLIC3 and ERK7 are not yet clear. One possibility is that ERK7 mediates phosphorylation of CLIC3 and/or chloride ion channel conductance. While our results suggest CLIC3 is not directly phosphorylated by ERK7, it is possible that the MAP kinase may facilitate association of CLIC3 with other key components or enzymes. Since ERK7 can inhibit DNA synthesis, it is also possible that CLIC3 is linked to a growth regulatory system, either by competing with growth regulatory proteins for binding to ERK7 or by facilitating chloride ion transport, leading to changes in intracellular pH and osmolarity.

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