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Identification of a Novel Member of the Chloride Intracellular Channel Gene Family (CLIC5) That Associates with the Actin Cytoskeleton of Placental Microvilli

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The chloride intracellular channel (CLIC) gene family has been implicated in chloride ion transport within various subcellular compartments. We report here the molecular, biochemical, and cellular characterization of a new member of this gene family termed CLIC5. CLIC5 was isolated from extracts of placental microvilli as a component of a multimeric complex consisting of several known cytoskeletal proteins, including actin, ezrin, α-actinin, gelsolin, and IQGAP1. We cloned human cDNAs and generated antibodies specific for CLIC5, CLIC1/NCC27, and CLIC4/huH1/p64H1. CLIC5 shares 52–76% overall identity with human CLIC1, CLIC2, CLIC3, and CLIC4. Northern blot analysis showed that CLIC5 has a distinct pattern of expression compared with CLIC1 and CLIC4. Immunoblot analysis of extracts from placental tissues demonstrated that CLIC4 and CLIC5 are enriched in isolated placental microvilli, whereas CLIC1 is not. Moreover, in contrast to CLIC1 and CLIC4, CLIC5 is associated with the detergent-insoluble cytoskeletal fraction of microvilli. Indirect immunofluorescence microscopy revealed that CLIC4 and CLIC5 are concentrated within the apical region of the trophoblast, whereas CLIC1 is distributed throughout the cytoplasm. These studies suggest that CLIC1, CLIC4, and CLIC5 play distinct roles in chloride transport and that CLIC5 interacts with the cortical actin cytoskeleton in polarized epithelial cells.

INTRODUCTION

Chloride channels are found on the plasma membrane of virtually all eukaryotic cells and play important roles in the maintenance of water and electrolyte balance, in the regulation of cell volume, and in the control of membrane potential (Jentsch, 1994). In polarized epithelial cells, proper chloride transport is essential for various absorptive and secretory processes (Begenisich and Melvin, 1998). Several heritable human diseases have been attributed to abnormal transport of chloride ions, including congenital myotonias (Koch *et al.*, 1992), kidney stone disease (Lloyd *et al.*, 1996), and cystic fibrosis (Riordan *et al.*, 1989).

In addition to their role in chloride transport at the plasma membrane, chloride channels have also been identified in various intracellular organelles, such as endosomes and the Golgi complex, where they are thought to play a physiological role in regulating the lumenal pH of these compartments (Al-Awqati, 1995). At least one class of molecules has been implicated in intracellular chloride transport. These molecules include members of the bovine p64 chloride channel gene family, which reside in various intracellular compartments. The bovine chloride channel protein p64 was originally isolated from kidney cortex vesicles and apical membranes of trachea. Biochemical and electrophysiological studies led to its assignment as a chloride channel protein (Landry *et al.*, 1989, 1993; Redhead *et al.*, 1992; Edwards *et al.*, 1998). Immunocytochemical studies have shown that p64 is localized to the plasma membrane and to membrane vesicles within the cytoplasm (Redhead *et al.*, 1992). However, expression of bovine p64 in *Xenopus laevis* oocytes results in the incorporation of p64 into microsomes but not the plasma membrane. These observations led to the hypothesis that p64 functions to facilitate the acidification of certain intracellular organelles by offsetting the electrical potential generated by proton ATPases (Redhead *et al.*, 1992; Landry *et al.*,

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Abbreviations used: CLIC, chloride intracellular channel; TBS, Tris-buffered saline.

1993). Consistent with this view, the ruffled border of the osteoclast plasma membrane expresses a closely related chloride channel, p62, that has been proposed to play a role in bone resorption by regulating acid transport (Schlesinger *et al.*, 1997).

Recently, several related human genes that share striking homology with the C-terminal half of bovine p64 have been cloned. These genes constitute a protein family called CLIC (chloride intracellular channel). The first member identified, CLIC1/NCC27, was originally localized in cell nuclei (Valenzuela *et al.*, 1997) and subsequently was shown to be enriched in the brush border of proximal tubule cells (Tulk and Edwards, 1998). CLIC1 shares 57% identity with bovine p64 (Valenzuela *et al.*, 1997). CLIC2/XAP121 was discovered while mapping transcripts derived from the telomeric region of chromosome Xq28 and shares 60% overall identity with CLIC1 (Heiss and Poustka *et al.*, 1997). CLIC3, which is 48–49% identical to CLIC1 and CLIC2, was identified in a two-hybrid assay as a binding partner for the C-terminal tail of ERK7, a member of the MAPK family (Qian *et al.*, 1999). In the same study, epitope-tagging experiments indicated that CLIC3 is localized predominantly in the nucleus. CLIC4/huH1/p64H1 was recently identified (Chuang *et al.*, 1999; Edwards, 1999) as the human homologue of a rat brain protein termed p64H1 (Duncan *et al.*, 1997) and shares 67, 66, and 49% identity with CLIC1, CLIC2, and CLIC3, respectively. Although rat brain p64H1 has been colocalized with markers for the endoplasmic reticulum in transfected cells (Duncan *et al.*, 1997), in another study it was localized to the large dense-core vesicles of rat hippocampal neurons (Chuang *et al.*, 1999). Human CLIC4 is enriched in the apical region of proximal tubule cells (Edwards, 1999). In addition, the mouse homologue of CLIC4, called mc3s5/mtCLIC, was recently localized to the mitochondria and cytoplasm of keratinocytes (Fernandez-Salas *et al.*, 1999). Furthermore, the expression of mc3s5/mtCLIC is regulated by p53 and tumor necrosis factor α. Various electrophysiological studies have shown that several members of this gene family, including bovine p64 (Landry *et al.*, 1989, 1993; Edwards *et al.*, 1998), CLIC1 (Valenzuela *et al.*, 1997), CLIC3 (Qian *et al.*, 1999), and rat brain p64H1 (Duncan *et al.*, 1997), play a role in chloride transport. The different subcellular distributions of these channels suggest that members of this gene family may have related but distinct functions, although direct comparisons between individual members have not yet been reported.

In this study, we describe an affinity chromatography assay in which immobilized GST fusion proteins containing the C terminus of ezrin were used to isolate endogenous proteins from human placental microvilli. Ezrin, a major membrane-cytoskeleton linking protein of placental microvilli, contains an F-actin–binding site in its extreme C terminus. However, this binding site is masked in full-length ezrin through an intramolecular interaction with its own N terminus. Thus, we reasoned that proteins that associate with the C terminus of ezrin or actin itself could be isolated with the use of truncated forms of ezrin that lack the N-terminal domain. We document the identification and cloning of a novel member of the CLIC family of chloride channel proteins that specifically associates with the cytoskeleton of placental microvilli.

MATERIALS AND METHODS

Materials

Constructs for the GST-ezrin fusion proteins were made and characterized in a previous study (Gary and Bretscher, 1995). Fusion proteins were expressed and purified from the protease-deficient host strain BL21 as described (Gary and Bretscher, 1995). Human placenta was obtained from consenting patients at Tompkins Community Hospital (Ithaca, NY). Antibodies to ezrin (Bretscher, 1989), α-actinin (Bretscher and Weber, 1978), and IQGAP1 (Hart *et al.*, 1996) have been described. Mouse mAbs against human placental alkaline phosphatase (clone 8B6) and rat protein disulfide isomerase (clone RL77) were purchased from Sigma Chemical (St. Louis, MO) and Affinity Bioreagents (Golden, CO), respectively.

Actin-binding Assays

Rabbit skeletal muscle actin was purified as described previously (MacLean-Fletcher and Pollard, 1980). Purified GST fusion proteins were dialyzed against PBS, and the concentrations were determined by a Bio-Rad (Hercules, CA) protein assay. All proteins were centrifuged at 100,000 $\times$ *g* for 15 min immediately before use. For high-speed cosedimentation assays, 2 μM GST fusion protein was mixed with 8 μM G-actin in the presence of 5 mM $MgCl_2$ and 1 mM ATP, incubated for 80 min at room temperature, and then centrifuged at 100,000 $\times$ *g* for 10 min. Equal volumes of supernatants and pellets were boiled in 5$\times$ Laemmli sample buffer and resolved by 12% SDS-PAGE. For electron microscopic analysis, the mixtures were diluted 10-fold, spotted onto carbon-coated Formvar grids, and stained by the addition of 1% uranyl acetate. Specimens were examined and photographed at 80 kV with a Philips (Mahwah, NJ) 301 electron microscope.

Affinity Chromatography Assays

Purified GST fusion proteins bound to glutathione–agarose at a concentration of 1–2 mg/ml were used as affinity beads. Human placental microvilli were isolated from fresh tissue (Berryman *et al.*, 1995), divided into aliquots at 2 mg protein/ml, and stored at −70°C. Upon thawing, microvilli were centrifuged at 20,000 $\times$ *g* for 15 min at 4°C. Soluble extracts of microvilli were prepared by resuspending the pellet to the original volume with 1% Triton X-100, 20 mM HEPES, pH 7.4, 0.6 M KCl, 1 mM EDTA, 1 mM PMSF, 0.5 mM benzamidine, 1 μg/ml leupeptin, 1 μg/ml pepstatin A. After trituration and incubation for 10 min at room temperature, the extracts were centrifuged at 100,000 $\times$ *g* for 20 min at 4°C. The supernatant was removed and diluted with 6 volumes of ice-cold 20 mM HEPES, pH 7.4, 7 mM NaCl, 1 mM PMSF, 0.5 mM benzamidine and then supplemented with 100 μM sodium orthovanadate and 10 mM $MgCl_2$. One volume of extract was mixed gently for 2–3 h at room temperature with 20 μl of affinity beads in the presence of 1 mM ATP to promote actin polymerization. In some experiments, purified skeletal muscle actin (1 μM) was added to the extract. Beads were washed five times with 20 mM HEPES, 100 mM NaCl, 0.1% Triton X-100, and bound proteins were eluted by boiling in 2$\times$ Laemmli sample buffer.

Protein Purification and Microsequencing

The affinity chromatography assay was scaled up ~100-fold to isolate proteins for sequence analysis. Beads containing GST-ezrin 475–586 were incubated batchwise with microvillus extract, washed, and eluted by incubation for 10 min at room temperature with 1 bed volume of 20 mM HEPES, pH 7.4, 2 M NaCl, 0.1% Triton X-100. Two additional elutions were performed with the use of 1 M

NaCl. The eluates were pooled, desalted, and concentrated in a Centricon 30 (Millipore, Bedford, MA). Proteins were boiled after the addition of 5× Laemmli sample buffer, resolved by SDS-PAGE, and blotted to polyvinylidene difluoride. The membrane was stained with Ponceau-S (Sigma Chemical), and the regions containing the 90-, 55-, and 32-kDa bands were excised and washed with distilled water. Subsequent sample processing was performed at the Cornell BioResource Center (Ithaca, NY). Approximately 2.5 μg of each protein was digested in situ with endoproteinase lys-C and then subjected to two-dimensional fractionation by HPLC. Peak fractions were analyzed by mass spectrometry and selected for automated microsequence analysis. Peptide sequences were used to search the National Center for Biotechnology Information database with the use of the BLAST program (Altschul *et al.*, 1990).

cDNA Cloning

The Human Gene Index at The Institute for Genomic Research (Rockville, MD) was searched with the use of the peptide sequences corresponding to the 32-kDa protein, and a cDNA clone whose predicted coding sequence matched the query was obtained from the American Type Culture Collection (Rockville, MD). The insert cDNA was sequenced in its entirety to yield a predicted partial ORF of 157 amino acids followed by a stop codon. A PCR product corresponding to the predicted coding sequence was used to generate a probe to screen a human placental cDNA library (λTriplEx, Clontech, Palo Alto, CA). The PCR product was made with the use of primers 5′-ACCTTGACCCCTGAAAAGTAC-3′ and 5′-GGATCGGCTGAGGCGTTTGGC-3′ and was labeled with [α-^{32}P]dCTP by random priming (RadPrime DNA labeling system, Life Technologies, Gaithersburg, MD). Hybridization was done at moderate and high stringency, and several individual clones were isolated and sequenced in both directions. DNA and protein sequences were edited and aligned with the use of the software programs EDITSEQ and MEGALIGN, respectively (DNASTAR, Madison, WI).

Northern Hybridizations

A commercial human multiple-tissue Northern blot was purchased (Clontech). The blot was probed sequentially with antisense DNAs corresponding to CLIC5, CLIC4, and CLIC1. Probes were labeled with [α-^{32}P]dATP by linear PCR with the use of template DNA corresponding to each of the predicted ORFs (Strip-EZ PCR kit, Ambion, Austin, TX). As a control, a commercial β-actin probe was labeled with [^{32}P]dATP with the use of the RadPrime DNA labeling system. Probes were diluted in ULTRAhyb (Ambion), incubated under high-stringency conditions, and stripped from the blot as recommended by the manufacturer.

Production of Recombinant Proteins

The cDNA sequences encoding CLIC5, CLIC4, and CLIC1 were used to produce proteins containing a six-histidine tag at their N termini as follows. The sequences encoding residues 1–251 of CLIC5, 1–253 of CLIC4, and 1–249 of CLIC1 were amplified by PCR with the use of primers that generated *Bam*HI and *Hin*dIII, *Bam*HI and *Kpn*I, and *Bam*HI and *Pst*I sites at their ends, respectively. The products were subcloned into pQE30 (Qiagen, Chatsworth, CA). To generate untagged versions of each protein, the coding sequences were amplified by PCR with the use of primers that introduced *Sph*I and *Hin*dIII sites at their ends and then were subcloned into pQE70 (Qiagen). This changed the second residue in CLIC5 and CLIC4 to leucine and introduced an additional leucine residue immediately after the initiating methionine in CLIC1. The untagged recombinant sequences were free of PCR errors, as determined by nucleotide sequencing. Proteins were expressed and total bacterial lysates were prepared as described previously (Reczek *et al.*, 1997).

Antibodies

To prepare antigens corresponding to CLIC5, CLIC4, and CLIC1, six histidine-tagged recombinant proteins were purified under denaturing conditions. Induced cells were resuspended in buffer A (6 M guanidine-HCl, 0.1 M sodium phosphate, 0.01 M Tris, pH 8.0) and stirred for 1 h at room temperature. The cells were further disrupted by sonication (Branson Sonifier, Danbury, CT) and centrifuged at 40,000 × *g* for 10 min at 15°C. The supernatant was mixed for 1 h at room temperature with 4 ml of iminodiacetic acid Sepharose 6B (Sigma Chemical) that had been charged with 50 mM $NiSO_4$ and equilibrated in buffer A. The slurry was poured into a column and washed sequentially with 50 ml of buffer A, 50 ml of buffer B (8 M urea, 0.1 M sodium phosphate, 0.01 M Tris, 0.1% Triton X-100, 5 mM β-mercaptoethanol, pH 8.0), and 50 ml of buffer C (buffer B at pH 6.3). Proteins were eluted with buffer D (buffer B at pH 4.0), and 2-ml fractions were collected and immediately neutralized by the addition of 1 M Tris base. Peak fractions were pooled, boiled in 2× Laemmli sample buffer, and run on 12% preparative SDS-PAGE. Bands were visualized by brief staining with Coomassie blue R-250 and excised, and the proteins were electroeluted and dialyzed extensively against 150 mM NaCl, 0.1% SDS. Antibodies were raised in rabbits at the Center for Research Animal Resources at the Cornell University College of Veterinary Medicine (Ithaca, NY). High-titer antisera were obtained for all three antigens and were designated B-132 (CLIC5), B-134 (CLIC4), and B-121 (CLIC1). To prepare monospecific antibodies, affinity columns were prepared with the use of purified recombinant histidine-tagged proteins with minor modifications to the method described previously (Bretscher, 1983). Serum from B-132, B-134, and B-121 was first passed through columns containing CLIC4, CLIC5, and CLIC5, respectively. Each cross-adsorbed serum was then affinity purified by passage over a column containing the cognate antigen.

Fractionation of Placental Tissue

A human placental extract was prepared by homogenization of frozen tissue in 2 volumes of 10 mM Tris, pH 7.6, 150 mM NaCl, 5 mM EDTA, 1 mM DTT, 1 mM PMSF, 0.5 mM benzamidine with the use of a Waring (New Hartford, CT) blender. The homogenate was passed through a 1000-mesh nylon screen and then centrifuged at 10,000 × *g* for 15 min at 4°C. The supernatant, representing whole placental tissue, was boiled after the addition of 2× Laemmli sample buffer. To obtain a soluble fraction, the low-speed supernatant was recentrifuged at 100,000 × *g* for 20 min at 4°C, followed by the addition of 2× Laemmli sample buffer and boiling. Placental membranes were prepared by homogenization of frozen tissue in 5 volumes of cold 10 mM Tris, pH 7.4, 0.32 M sucrose, 1 mM DTT, 5 mM EDTA, 2 mM NaN_3, 1 mM PMSF, 0.5 mM benzamidine, 1 μg/ml leupeptin, 1 μg/ml pepstatin A. The homogenate was filtered through nylon mesh and centrifuged at 900 × *g* for 10 min at 4°C. The supernatant was centrifuged at 100,000 × *g* for 1 h to pellet membranes. Membranes were resuspended in 10 mM HEPES, pH 7.4, 75 mM NaCl, 20 mM EGTA, 15 mM $MgCl_2$, and protease inhibitors and then recentrifuged. Washed membranes, which would be expected to contain both microvillar and nonmicrovillar membranes as well as associated cytoskeletal elements, were resuspended in the same buffer at a concentration of 5 mg/ml and boiled after the addition of 2× Laemmli sample buffer. Membrane and cytoskeletal fractions were prepared from isolated placental microvilli as described previously (Berryman *et al.*, 1995).

SDS-PAGE and Immunoblotting

Proteins were resolved by SDS-PAGE according to standard procedures (Laemmli, 1970) and visualized by staining with Coomassie blue R-250. For immunoblots, proteins were transferred to polyvinylidene difluoride membranes (Immobilon-P, Millipore, Bedford, MA) with the use of a semidry electroblotter (Integrated Separation Systems, Hyde Park, MA). Blots were blocked with 10% nonfat

milk, and antibodies were diluted in 1% milk/Tris-buffered saline (TBS) containing 0.1% Tween 20. Bound antibodies were detected with an appropriate peroxidase-labeled secondary reagent. Reactive bands were visualized with the use of ECL (Amersham, Arlington Heights, IL).

Immunofluorescence Microscopy

Cryosections of human placenta (6 μm) were fixed for 10 min in 2% formaldehyde/PBS followed by permeabilization with absolute ethanol for 5 min at −20°C. Free aldehydes were quenched by treatment with 150 mM glycine/TBS. The sections were rinsed in distilled water and then boiled for 1–2 min in antigen-unmasking solution (Vector Laboratories, Burlingame, CA). After rinsing briefly with distilled water, sections were blocked with 5% goat serum/TBS and stained with primary antibodies diluted in blocking buffer. Sections were rinsed with TBS, stained with FITC-conjugated anti-rabbit immunoglobulin G (ICN Biomedicals, Costa Mesa, CA), rinsed again with TBS, and then mounted in Vectashield mounting medium (Vector Laboratories). Primary antibodies were omitted in controls. Sections were viewed with a Zeiss (Thornwood, NY) Axiovert 100-TV fluorescence microscope, and images were acquired with the use of Metamorph imaging software (Universal Imaging, West Chester, PA).

RESULTS

Binding of Skeletal Muscle F-Actin to the C Terminus of Ezrin In Vitro

Previous studies have indicated that the C-terminal domain of ezrin contains a binding site for F-actin (Turunen *et al.*, 1994; Pestonjamasp *et al.*, 1995) and that this site is masked in the native ezrin molecule (Gary and Bretscher, 1995). In contrast, Roy *et al.* (1997) suggested that binding of F-actin to the C-terminal region of ezrin is dependent on residues in the N-terminal domain. In this report, we used a GST fusion protein that contains only the C-terminal 30 residues of ezrin. Thus, our initial experiments tested the ability of this fusion protein to bind to F-actin in vitro (Figure 1). We found that GST-ezrin 556–586, but not GST alone, bound to rabbit skeletal muscle F-actin in high-speed (>100,000 × *g*) cosedimentation assays (Figure 1A). In addition, F-actin sedimented at low speeds (16,000 × *g*) after incubation with GST-ezrin 556–586, suggesting that this fusion protein bundles actin filaments (our unpublished data). This was confirmed by electron microscopic analysis of mixtures of F-actin and GST-ezrin 556–586, which revealed the presence of large bundles of parallel actin filaments (Figure 1B). Although the biochemical evidence shows that ezrin is responsible for binding to F-actin, the observed cross-linking activity likely results from the fact that GST forms stable dimers (Parker *et al.*, 1990), which would create two F-actin–binding sites per molecule in the assays described above.

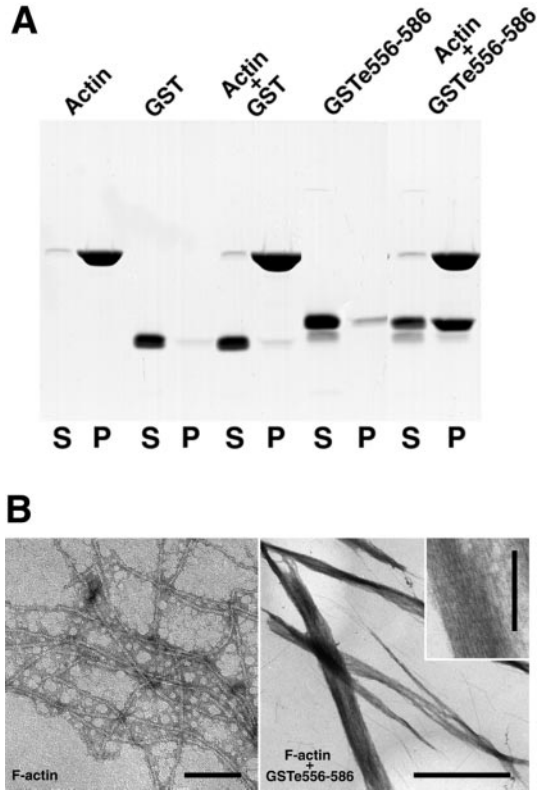

Figure 1. Direct binding of skeletal muscle actin to the C-terminal 30 residues of ezrin. (A) Purified actin or buffer alone was incubated with GST or GST-ezrin fusion protein (GSTe556–586), followed by ultracentrifugation. Fractions corresponding to the supernatant (S) and pellet (P) were resolved by SDS-PAGE and visualized with Coomassie blue staining. (B) F-actin incubated alone (bar, 0.1 μm) or in combination with GST-ezrin 556–586 (bar, 1.0 μm) was visualized by negative-stain electron microscopy as described in MATERIALS AND METHODS. The inset shows part of a bundle of parallel actin filaments at high magnification (bar, 0.1 μm).

Identification and Purification of a Cytoskeleton-associated 32-kDa Protein from Soluble Extracts of Isolated Placental Microvilli

Next, we established conditions for the binding of F-actin to immobilized GST-ezrin 556–586 in an affinity chromatography assay. The GST-ezrin fusion protein was incubated with actin in the presence of ATP, followed by washing and elution of bound proteins with SDS. At relatively low concentrations of actin (40 μg/ml), specific binding of actin was seen on beads containing GST-ezrin 556–586 (Figure 2, compare lanes 4 and 8). However, there are several noteworthy technical considerations concerning the specificity of F-actin binding in this assay. First, although the concentration of G-actin must exceed the critical concentration for polymerization, excessive concentrations can result in nonspecific binding, presumably because filaments polymerize and become trapped within the bead matrix. Second, bound F-actin can dissociate from the beads during washing, especially in the absence of F-actin cross-linking proteins that would otherwise help stabilize the bound filaments. Thus, a combination of relatively high concentrations of actin and the presence of F-actin cross-linking proteins can lead to increased background binding of cytoskeleton-associated proteins to control beads (Figure 2, compare lanes 4 and 5).

To identify placental microvillus proteins that associate with the C terminus of ezrin or F-actin, we mixed immobilized GST-ezrin 556–586 with detergent-soluble microvillus extracts. In this assay, the soluble actin present as an endogenous component of microvilli was expected to bind to the affinity matrix. Microvillus extracts were found to contain

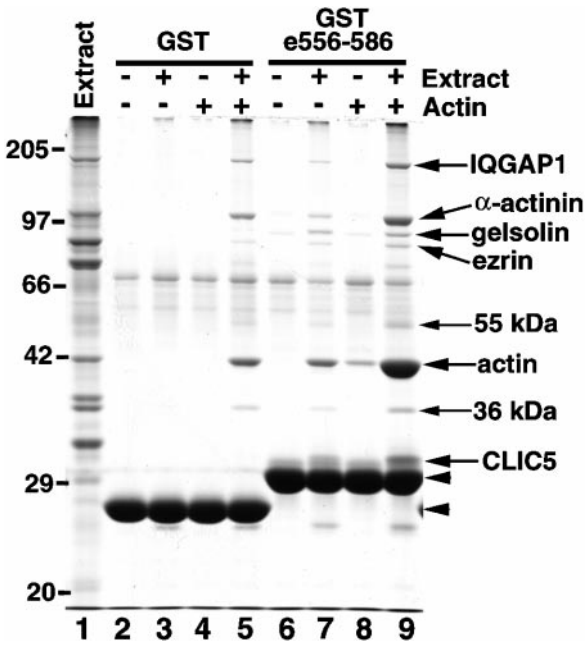

Figure 2. Identification of placental microvillus proteins that associate with the C-terminal 30 residues of ezrin. Glutathione–agarose affinity beads containing GST or GST-ezrin 556–586 (GSTe556–586) were incubated with buffer alone, microvillus extracts, or actin. After washing, bound proteins were eluted, run on 12% SDS-PAGE, and stained with Coomassie blue. The mobilities of the fusion proteins are indicated by arrowheads. The mobilities of protein standards are indicated on the left in kilodaltons.

several distinct polypeptides having apparent molecular masses of 190, 105, 90, 82, 55, 42, and 32 kDa (CLIC5) that specifically associated with the C terminus of ezrin (Figure 2, lane 7). In addition, the proteins showed enhanced binding in microvillus extracts that were supplemented with exogenous actin (Figure 2, compare lanes 7 and 9). In other experiments, these proteins collectively bound to GST-ezrin fusion proteins expressing residues 369–586, 475–586, 480–586, 531–586, and 556–586, all of which contain an intact F-actin–binding site. In contrast, the collection of proteins was greatly reduced or absent in experiments that used GST alone or GST-ezrin constructs encoding residues 1–586, 1–369, 1–440, 1–514, and 475–584, all of which either have masked C termini or are missing residues suggested to be important for F-actin binding (Turunen *et al.*, 1994). Note the presence of several bacterially derived background bands, especially one that migrates slightly faster than CLIC5, by comparing lanes 6 and 7 in Figure 2. In addition, CLIC5 is not evident in control GST beads incubated with microvillus extract and exogenous actin, even though some actin and its associated proteins are trapped by these beads (Figure 2, lane 5). The binding specificity of the 36-kDa polypeptide seen in Figure 2 varied from experiment to experiment. Thus, this 36-kDa protein, which likely represents a member of the annexin protein family (Edwards and Booth, 1987), was not pursued further in this study. These results demonstrate that the C-terminal 30 residues of ezrin can retain a multimeric protein complex consisting of actin and associated proteins.

The identity of each polypeptide was determined by immunoblot analysis or protein sequencing. Immunoblotting with specific antibodies revealed that the 190-, 105-, 82-, and 42-kDa bands represented IQGAP1, α-actinin, ezrin, and actin, respectively (our unpublished data). This was consistent with their mobility on SDS-PAGE and the fact that these proteins are known constituents of the actin cytoskeleton. Two peptide sequences were derived from the 90-kDa band, VSNGAGTMSVSLVADEN and QTQVSVLPEGGET, and database searches showed a perfect match to residues 303–319 and 374–386, respectively, of human gelsolin (Kwiatkowski *et al.*, 1986). One peptide sequence was derived from the 55-kDa band, which matched a sequence in the N-terminal half of ezrin, suggesting that this band is a degradation product of ezrin. Two peptide sequences were derived from the 32-kDa band, IEEFLEETLTPEKYPK and HRESNTAGIDIFSK (Figure 3). Database searches showed that highly related sequences are present in p64 (GenBank accession number L16547; Landry *et al.*, 1993), a bovine chloride channel protein originally isolated from kidney cortex vesicles and apical membranes of trachea (Landry *et al.*, 1989).

The human Expressed Sequence Tag database was found to contain a cDNA clone (EST33668; GenBank accession number AA329998) encoding part of the first and all of the second peptide sequence from the 32-kDa protein. Radiation hybrid mapping data from the Tentative Human Consensus report indicate that the corresponding gene is located on human chromosome 6. The insert contained a partial predicted ORF encoding 157 amino acid residues that share 94% identity with the C-terminal 157 residues of bovine p64. In addition, the insert was 65, 60, 50, and 70% identical to the C-terminal halves of CLIC1/NCC27 (Valenzuela *et al.*, 1997), CLIC2 (Heiss and Poustka, 1997), CLIC3 (Qian *et al.*, 1999), and CLIC4/huH1/p64H1 (Chuang *et al.*, 1999; Edwards, 1999), respectively. Therefore, the 32-kDa protein represents a novel member of the bovine p64 chloride channel family that we refer to as CLIC5 on the basis of recently described nomenclature (Edwards, 1999; Qian *et al.*, 1999).

The most abundant microvillus proteins that bound to GST-ezrin 556–586 (Figure 2, lane 7) were determined by densitometry to be CLIC5 and actin (0.88:1.0 molar ratio, CLIC5:actin). This suggests that CLIC5 might interact directly with actin or with the C terminus of ezrin.

Isolation and Analysis of cDNAs Encoding Three Related Human Chloride Channels and Comparative Northern Blots

To obtain a cDNA encoding the entire ORF of CLIC5, we screened a commercially available human placental cDNA library with the use of the DNA sequence from EST33668 as a probe. Under moderate-stringency conditions for hybridization, we isolated several clones corresponding to another member of the CLIC family, CLIC1/NCC27. The nucleotide sequence encoding the ORF of CLIC1 was identical to that reported previously (Tulk and Edwards, 1998). In addition, we isolated a cDNA clone of 1934 base pairs that apparently corresponds to the sequence of CLIC4/huH1/p64H1 (GenBank accession numbers AF097330 and AF109196). However, the cDNA we isolated had four nucleotides that differed from two independent reports on the sequence of

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                                CGGATCCTGTGACACCTCCGGGCAGCCCGGCACTTGTTGCTCCCACGACCTGTTGTCATT 60
CCCTTAACCCGGCTTTCCCCGCCTCCTCCCGGCTTCGCTCCTTTTCATGTGAGCATCTGGGACACTGATCTCTCAGACC 139
CCGCTGCTCGGGCTGGAGAATAGATGGTTTTGTGAAAAATTAAACACCGCCCTGAAGAGGAGCCCCGCTGGGCAGCGGC 218
AGGAGCGCAGAGTGCTGGCCCAGGTGCTGCAGAGGTGGCGCCTCCCCGGCCCGGGACGGTAGCCCCGGGCGCCAACGGC 297

ATG ACA GAC TCG GCG ACA GCT AAC GGG GAC GAC AGT GAC CCC GAG ATC GAG CTC TTT GTG 357
M   T   D   S   A   T   A   N   G   D   D   S   D   P   E   I   E   L   F   V   20

AAG GCT GGA ATC GAT GGA GAA AGC ATC GGC AAC TGT CCT TTC TCT CAG CGC CTC TTC ATG 417
K   A   G   I   D   G   E   S   I   G   N   C   P   F   S   Q   R   L   F   M   40

ATC CTC TGG CTG AAA GGA GTC GTG TTC AAT GTC ACC ACT GTG GAT CTG AAA AGA AAG CCA 477
I   L   W   L   K   G   V   V   F   N   V   T   T   V   D   L   K   R   K   P   60

GCT GAC CTG CAC AAC CTA GCC CCC GGC ACG CAC CCG CCC TTC CTG ACC TTC AAC GGG GAC 537
A   D   L   H   N   L   A   P   G   T   H   P   P   F   L   T   F   N   G   D   80

GTG AAG ACA GAC GTC AAT AAG ATC GAG GAG TTC CTG GAG GAG ACC TTG ACC CCT GAA AAG 597
V   K   T   D   V   N   K   I   E   E   F   L   E   E   T   L   T   P   E   K   100

TAC CCC AAA CTG GCT GCA AAA CAC CGG GAA TCC AAC ACA GCG GGC ATC GAC ATC TTT TCC 657
Y   P   K   L   A   A   K   H   R   E   S   N   T   A   G   I   D   I   F   S   120

AAG TTT TCT GCC TAC ATC AAA AAT ACC AAG CAG CAG AAC AAT GCT GCT CTT GAA AGA GGC 717
K   F   S   A   Y   I   K   N   T   K   Q   Q   N   N   A   A   L   E   R   G   140

CTA ACC AAG GCT CTA AAG AAA TTG GAT GAC TAC CTG AAC ACC CCT CTA CCA GAG GAG ATT 777
L   T   K   A   L   K   K   L   D   D   Y   L   N   T   P   L   P   E   E   I   160

GAC GCC AAC ACT TGT GGG GAA GAC AAG GGG TCC CGG CGC AAG TTC CTG GAT GGG GAT GAG 837
D   A   N   T   C   G   E   D   K   G   S   R   R   K   F   L   D   G   D   E   180

CTG ACC CTG GCT GAC TGC AAT CTG TTG CCC AAG CTC CAT GTG GTC AAG ATT GTG GCC AAG 897
L   T   L   A   D   C   N   L   L   P   K   L   H   V   V   K   I   V   A   K   200

AAA TAC CGC AAC TAT GAT ATC CCG GCT GAG ATG ACA GGC CTG TGG CGG TAC CTC AAG AAC 957
K   Y   R   N   Y   D   I   P   A   E   M   T   G   L   W   R   Y   L   K   N   220

GCC TAT GCC CGT GAT GAG TTC ACC AAC ACC TGT GCA GCT GAC AGT GAG ATC GAG TTG GCC 1017
A   Y   A   R   D   E   F   T   N   T   C   A   A   D   S   E   I   E   L   A   240

TAC GCT GAT GTC GCC AAA CGC CTC AGC CGA TCC TGA                                 1053
Y   A   D   V   A   K   R   L   S   R   S   *                                   251

GCACAGCCATTTTGCCCCATCCCCGCTGCAGAAGGACTCAACCACTCCCCTAAGACTCCAGCTTCATAGACTCCTCTGT 1132
ATCACTGCCTTGAGGCGCACTTTTTATAATCAAGCCTCATCTTGCTGGTATCATGGGAACTCCAGCCTGCTATCTTTCA 1211
TGAAGGTCAGCACCATCCCTGGCCTCCTCACATAGGAATCTAGCAGAAATGATAGACACAGTCCACCTTTCGGCCGGCC 1290
AGCCTGATCTGGGCTCAGCATGTTTGGGGTCAGTCAGTGTTGGAGAGCCCACATATGGGATTGCCACTAGCTTCTTCTG 1369
CCAATATCAAAATACCTTCTCAGATGCTTTAGAAACATGCAACACCAACTCCTTTTCTACCCTCCTCTCTGTCCATACC 1448
TACAAGGCCAAGGACAAACGCCATCTTCATCCTTCTTAGAAAGAGATCTATTACCCCATTAGGGGAGACAGAGAGAGTG 1527
AATGGAGGAGTACCGAGCTGGCTATGGACTTGGGTGTCTGGCAAACACAGCTTCAGTCTCACTACTTCTGACACTCTGG 1606
TTATTGGGCACTAAGGGCCAGACTGGAAAGTCACTTGAGACACATTCTCAGTTTGTTGCAGTGCCAGGAATGCTGCGCT 1685
GCTGCTGCTGCGCACCTGGCCCATGCTGTCCCTGGCTTCCATGCCGTCCAGGCCCTGCCAGAAAAGGAAATTGGCATGC 1764
AATTCTAAACTGCAGTGACTGGGATGGGAGGGGAGGGGAGCAGTGTTGATGCCAAAATACCCACGGGGTCTACCAGCCA 1843
TGGGGTTTGCTTGCTTAGGAGTAGTTGTTTCAGAGGTGATTACAGGCCTGGGTTTGACTGTGCTTACCAATGAGTGGTT 1922
TTTGAGCTATGAGAAAGTGGATGGGAGTGGGAGGAGGAGAGATGGGTGAAGACAAAAGAGTTCTTTATGAGCCTCGATG 2001
TTCCCTGGTAAACTTTTAAAAAGGCCTTCTCTCATGATCTAAGTCTTGGACTGGTGGCATCATGTAACTGCTAACCTTA 2080
CAGTAAAAACCCAAGAATGGGTCAAAAATGTCTTCCCAGTTTCTCCAAGCTGCTTCTGGAATGCAGGTCTGTCGGCTGG 2159
GTGCTCTCCAGCAGCTGCTCCTGCCTGATTCAACTGTAGCCTGTAATGGGTAAAAGCCACATTTAGGAGGTGGTCTGAT 2238
CATAGAACACCTTAGGAAGAAAGTCCATGAGACTTTCTGACTAGGAAACCATGTGGTTTGAACTTGAAGAAAAATGTAG 2317
ACCCATCTGGGTTAATTTTCCTACAATCTGACTCAACTGCCAGGTGAAAAAAAAAAAAAAAAA               2380
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Figure 3. Nucleotide and deduced amino acid sequences of human CLIC5 cDNA. Residues corresponding to the two placental CLIC5 peptide sequences are underlined. This sequence is available from GenBank under accession number AF216941.

CLIC4 (Chuang *et al.*, 1999; Edwards, 1999), each of which resulted in a change in amino acid sequence. In our cDNA, amino acid residues R133, P134, Y225, and T233, each of which is conserved in rat (Duncan *et al.*, 1997), mouse (Fernandez-Salas *et al.*, 1999), and cow (Chuang *et al.*, 1999), were encoded by agg, cca, tac, and acc, respectively. Under high-stringency conditions for hybridization, we obtained a single clone that contained the sequence of the EST33668 clone and thus represents CLIC5. The cDNA sequence (GenBank accession number AF216941) and the derived amino acid sequence from the largest ORF are shown in Figure 3. The cDNA is composed of 2380 base pairs. The first ATG in the sequence occurs at nucleotide 298, and the surrounding sequence presents a loose consensus for a translation start site with a purine at position −3 (Kozak, 1991). The first in-frame stop codon occurs at position 1051, resulting in a CLIC5 cDNA that encodes a 251-amino acid protein with a predicted molecular mass of 28,113 Da.

An alignment of the CLIC5 protein sequence with that of CLIC4, CLIC3, CLIC2, CLIC1, and the C-terminal half of bovine p64 is shown in Figure 4A. CLIC5 displays 76, 52, 66, and 63% overall identity with CLIC4, CLIC3, CLIC2, and CLIC1, respectively. In addition, CLIC5 is 91% identical to residues 197–437 of bovine p64. However, in contrast to CLIC1–CLIC5, which align very well over their entire lengths, p64 contains an additional N-terminal domain consisting of 199 amino acid residues that are divergent from the other members of this family. Inspection of Kyte-Doolittle hydropathy plots (Kyte and Doolittle, 1982) showed that the two major hydrophobic domains of CLIC1–CLIC5 are highly conserved (Figure 4B), and these domains are also present in the C-terminal half of bovine p64 (Landry *et al.*, 1993). However, there are considerable differences among the sequences at the extreme N termini of CLIC1–CLIC5. A family tree was derived from the primary sequences of CLIC1–CLIC5 to show the degree of conservation between different members (Figure 4C). A search for known protein motifs revealed that these proteins have no apparent N-terminal signal peptide or consensus sequence for retention in the endoplasmic reticulum (KDEL). However, as indicated in Figure 4A, several highly conserved protein-targeting motifs were identified (Marks *et al.*, 1996; Owen and Evans, 1998). Three tyrosine-based internalization signal motifs (YXXO) were found to be present at positions 101, 203, and 241 of CLIC5. The first two motifs are also conserved in bovine p64, CLIC1, CLIC2, and CLIC4, and the last motif is conserved among all family members. In addition, it is interesting to note that bovine p64, CLIC5, and CLIC4 all have conserved consensus sequences for phosphorylation by cAMP-dependent protein kinase at their extreme C termini.

A commercially available Northern blot containing purified poly(A)$^+$ RNA from various human tissues was used to compare the expression of CLIC5, CLIC4, and CLIC1 (Figure 5). The major CLIC5 transcript was 6.4 kilobases (kb), with minor transcript sizes of 3.8 and 2.3 kb. High levels of CLIC5 were detected in heart and skeletal muscle, and moderate levels were present in kidney, lung, and placenta. In addition to the information obtained by Northern blot analysis, Expressed Sequence Tag database searches revealed that cDNA clones representing CLIC5 are present in 12-wk human embryo, infant brain, and testis. A single transcript of 4.5 kb was seen for CLIC4 in all tissues examined, with the highest levels in heart, placenta, and kidney. Two transcripts of 1.2 and 1.4 kb were seen for CLIC1, which was abundant in heart, placenta, liver, kidney, and pancreas. Little or no CLIC1 was detected in skeletal muscle, lung, and brain.

Analysis of Recombinant CLIC5, CLIC4, and CLIC1 and Characterization of Monospecific Antibodies

We designed constructs encoding full-length untagged versions of CLIC5, CLIC4, and CLIC1 for expression in bacteria. Although the generation of these constructs resulted in minor amino acid changes in the extreme N terminus of each recombinant protein (see MATERIALS AND METHODS), the affect on the total molecular mass of each protein was negligible. Analysis of whole bacterial lysates by SDS-PAGE showed major induced bands having relative mobilities of 29 kDa for CLIC4, 31 kDa for CLIC1, and 32 kDa for CLIC5 (Figure 6A). We expressed and purified six histidine-tagged versions of CLIC5, CLIC4, and CLIC1 and used them to immunize rabbits for the production of antibodies. Monospecific antibodies were prepared by cross-adsorption of sera against an appropriate family member before affinity purification. The antibodies displayed remarkable specificity on immunoblots containing equal amounts of each of the untagged recombinant proteins (Figure 6B).

CLIC5 Is Associated with the Cytoskeleton of Isolated Placental Microvilli

As shown in the stained gel in Figure 2, we originally identified a novel 32-kDa polypeptide as a component of a multimeric protein complex that interacts with the C-terminal 30 residues of ezrin in the affinity chromatography assay. We assumed that this 32-kDa protein truly represented CLIC5 because untagged recombinant CLIC5 and the 32-kDa protein exhibited nearly identical mobilities on SDS-PAGE. Using antibodies specific to CLIC5, we verified that this 32-kDa protein represents CLIC5 in immunoblots containing the samples shown in Figure 2 (our unpublished data).

To further investigate the interaction of CLIC5, and to assess whether or not CLIC1 and CLIC4 could also interact with the cytoskeleton, the affinity chromatography assay was performed with the use of different GST-ezrin fusion proteins: GST-ezrin 475–586 and GST-ezrin 475–584 (Figure 7). Two previous studies have shown that residues in the extreme C terminus of ezrin play an important role in ezrin's binding to itself and to F-actin (Turunen *et al.*, 1994; Gary and Bretscher, 1995). Accordingly, we found that the GST-ezrin 475–584 fusion protein bound poorly to F-actin (our unpublished data). As shown in Figure 7 (A and B, lanes 4), we observed strong binding of CLIC5 and relatively low levels of CLIC4 binding to GST-ezrin 475–586. The ratios of immunoreactive bands in the starting material compared with what bound to the beads (compare lanes 1 and 4) indicate that CLIC5 has a much greater binding affinity than CLIC4 in this assay. In contrast, CLIC5 and CLIC4 showed little or no binding to GST alone or GST-ezrin 475–584, the construct lacking residues required for binding to the N-terminal domain of ezrin and to F-actin (Figure 7, lanes 2 and 3). As expected, α-actinin showed specific binding in

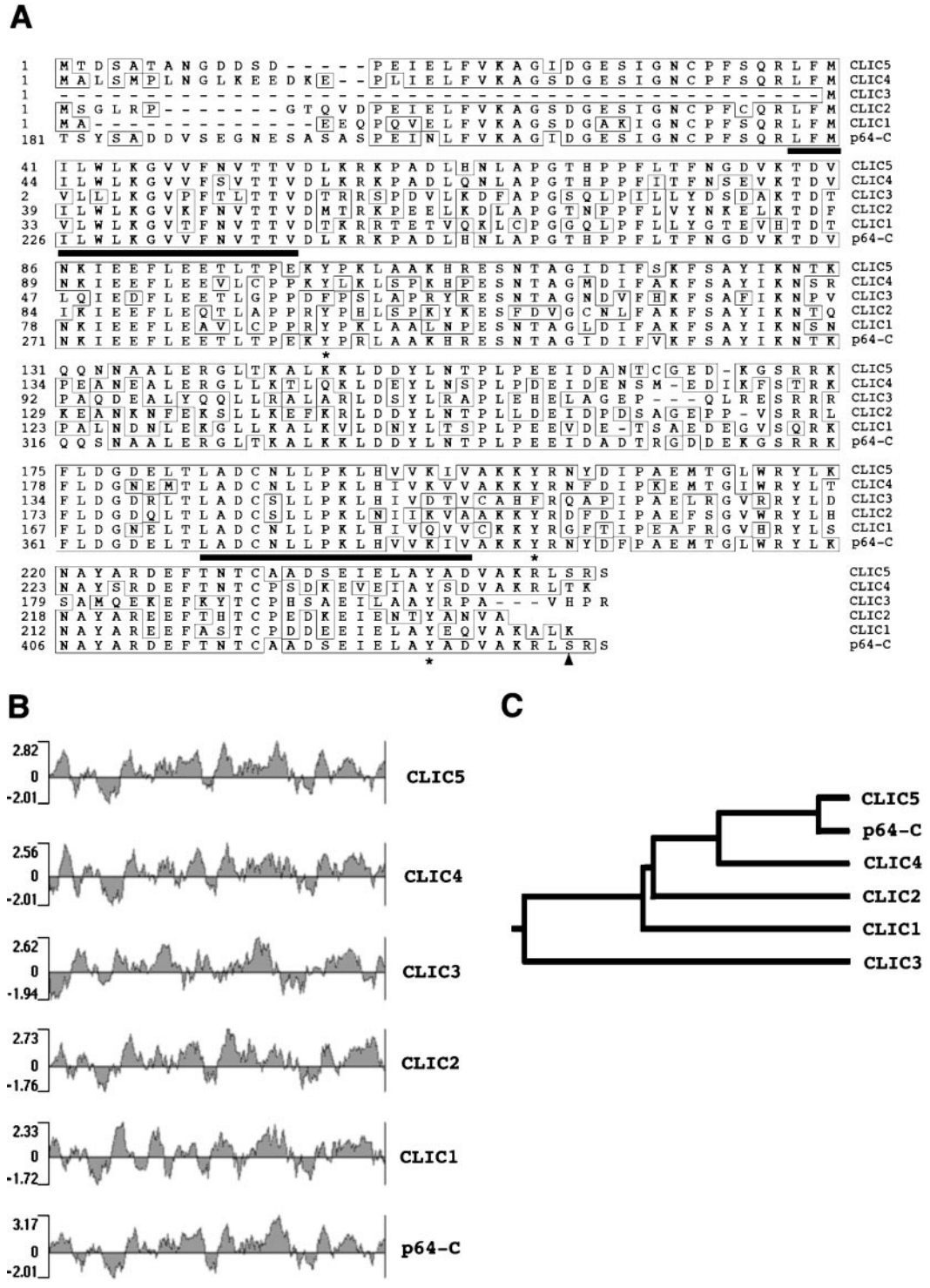

Figure 4. Human CLIC5 has homology to CLIC4, CLIC3, CLIC2, CLIC1, and the C-terminal half of bovine p64 (p64-C). (A) Sequence alignment. Amino acid residues are numbered on the left. Identities are boxed. The two conserved hydrophobic domains are underlined. Three highly conserved tyrosine residues are indicated by asterisks. The arrowhead indicates a potential phosphorylation site for cAMP-dependent protein kinase that is conserved in CLIC5, CLIC4, and p64-C. (B) Kyte-Doolittle hydrophobicity plots. (C) Dendrogram of CLIC proteins.

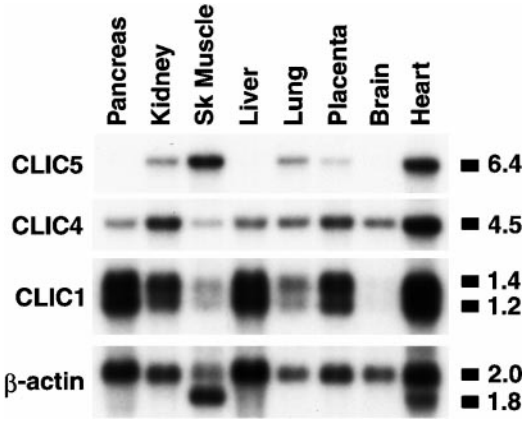

Figure 5. Comparison of the mRNA expression of CLIC5, CLIC4, and CLIC1 in human tissues. A commercially available Northern blot was probed sequentially with radiolabeled antisense DNA corresponding to the coding sequences of CLIC5, CLIC4, and CLIC1. A β-actin probe was used as a control. The sizes of the major transcripts are indicated on the right in kilobases.

this assay (Figure 7C). However, no binding of CLIC1 was observed (our unpublished data).

To assess the subcellular distribution of CLIC5, CLIC4, and CLIC1 in human placenta, tissue was fractionated biochemically and examined by immunoblotting (Figure 8). Both CLIC5 and CLIC4 were highly enriched in extracts of isolated microvilli over that seen in extracts of whole placental tissue (Figure 8A, compare lanes 1 and 4). In contrast, CLIC1 showed the reverse trend. As expected, placental alkaline phosphatase (PLAP) was found to be enriched in our microvillus preparations, whereas a marker for the endoplasmic reticulum, protein disulfide isomerase (PDI), was present at lower levels in microvilli than in whole placental extracts. By comparison of known amounts of CLIC proteins and whole microvillus extracts on immunoblots, we estimated that CLIC5 and CLIC4 account for ~0.7 and 0.2%, respectively, of total microvillus proteins. Interestingly, in contrast to CLIC5 and CLIC4, a substantial amount of CLIC1 was present in the soluble fraction of placental tissue (Figure 8B, lane 2), consistent with previous observations made by Tulk and Edwards (1998). In addition, a faint, slower-migrating, 32-kDa CLIC1-reactive band that was distinct from CLIC5 was seen in the membrane and microvillus fractions (Figure 8B, lanes 3, 4, and 6).

The association of CLIC5 with the cytoskeleton of placental microvilli was confirmed independent of the affinity chromatography assay by biochemical analysis of the purified structures. Cytoskeletal and membrane fractions of microvilli were prepared by conventional methods with the use of a nonionic detergent, and the relative amounts of CLIC5, CLIC4, and CLIC1 in these fractions were assayed by immunoblotting (Figure 8, A and B, lanes 5 and 6). Approximately 70% of CLIC5 was retained by the insoluble cytoskeletal residue, whereas CLIC1 and CLIC4 were completely detergent soluble under the conditions we used. The distinct fractionation profile for CLIC5 suggests that it associates with the cytoskeleton in vivo.

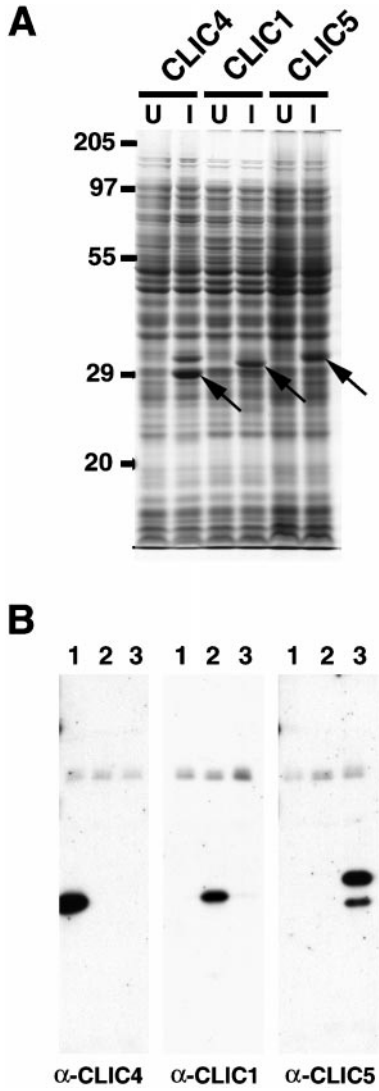

Figure 6. Expression of recombinant CLIC proteins and characterization of monospecific antibodies. (A) Expression of untagged CLIC4, CLIC1, and CLIC5. Bacterial extracts from uninduced (U) and induced (I) cultures were run on 12% SDS-PAGE and stained with Coomassie blue. The major induced bands are indicated by arrows. The mobility of standard proteins is indicated on the left in kilodaltons. (B) Immunoblot analysis of induced bacterial lysates expressing untagged CLIC4 (lanes 1), CLIC1 (lanes 2), and CLIC5 (lanes 3). Lysates containing equal amounts (20 ng) of the induced CLIC proteins were resolved by 12% SDS-PAGE, transferred to membranes, and probed with specific antibodies, as indicated at the bottom of each panel. Note that there is an additional induced band that migrates slower than CLIC4 in the lysate shown in A that is not recognized in the corresponding immunoblot in B. In addition, the band that migrates faster than CLIC5 in the CLIC5 immunoblot in B presumably represents a bacterially derived degradation product of CLIC5.

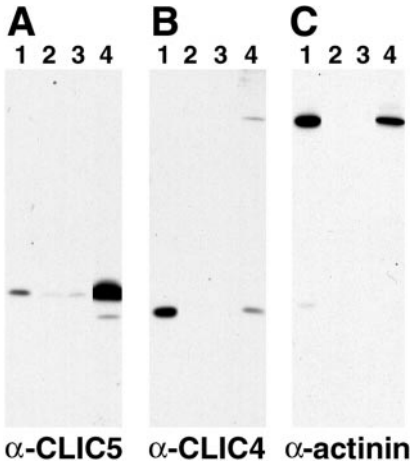

Figure 7. Association of CLIC5 with cytoskeletal components in the affinity chromatography assay. A soluble extract of placental microvilli (lanes 1) was incubated with glutathione–agarose affinity beads containing GST (lanes 2), GST-ezrin 475–584 (lanes 3), and GST-ezrin 475–586 (lanes 4). The beads were washed and eluted, and the bound proteins were run on 12% SDS-PAGE and blotted. (A) Blot probed with antibody against CLIC5. (B) Blot probed with antibody against CLIC4. (C) Blot probed with antibody against α-actinin.

Localization of Chloride Channels in Human Placenta

The subcellular distribution of CLIC5, CLIC4, and CLIC1 was studied by indirect immunofluorescence microscopy on cryosections of human placenta (Figure 9). All three proteins were expressed at the highest levels in the trophoblast epithelium. The staining pattern for CLIC5 and CLIC4 overlapped to a great extent (Figure 9, compare A and C). Both proteins showed intense staining on the apical surface of the trophoblast epithelium. A subtle distinction in staining between CLIC4 and CLIC5 was that CLIC4 appeared to have greater amounts of diffuse staining in the apical cytoplasm than CLIC5 (Figure 9D, arrows). In contrast, CLIC1 staining was not enriched on the apical surface but was seen throughout the cytoplasm of the trophoblast. At higher magnification, punctate staining was observed for CLIC1, which was often found near the basal surface of the trophoblast and within cells embedded in the underlying connective tissue (Figure 9F). In addition to the epithelium, all three proteins displayed low but variable levels of staining in cells associated with blood vessels. None of the CLIC family members we examined were localized to the nuclei of any cells in placenta. Unfortunately, our attempts to localize the proteins by immunoelectron microscopy were not successful.

DISCUSSION

We have identified CLIC5 as a putative chloride channel that associates with the actin cytoskeleton of the human placental syncytiotrophoblast. The name CLIC5 was chosen on the basis of the high sequence homology it shares with known members of the chloride intracellular channel gene family, including bovine p64 (Landry *et al.*, 1993), CLIC1/NCC27 (Valenzuela *et al.*, 1997), CLIC2 (Heiss and Poustka, 1997), CLIC3 (Qian *et al.*, 1999), and CLIC4/huH1/p64H1 (Chuang *et al.*, 1999; Edwards, 1999). By direct comparison with CLIC1 and CLIC4, we have demonstrated that CLIC5 has unique biochemical properties and a subcellular distribution that suggest that it functions at or near the apical plasma membrane in polarized epithelial cells.

Association with the Actin Cytoskeleton

We used the C-terminal domain of ezrin as an affinity matrix to identify a complex of cytoskeletal proteins that includes actin, α-actinin, gelsolin, IQGAP1, and CLIC5. Our initial experiments confirm that the isolated C-terminal domain of ezrin binds directly to purified skeletal muscle F-actin, a subject of some controversy (Bretscher, 1999). From these results, we infer that the immobilized C-terminal domain of ezrin binds directly to the endogenous actin derived from placental microvilli in the affinity chromatography assay.

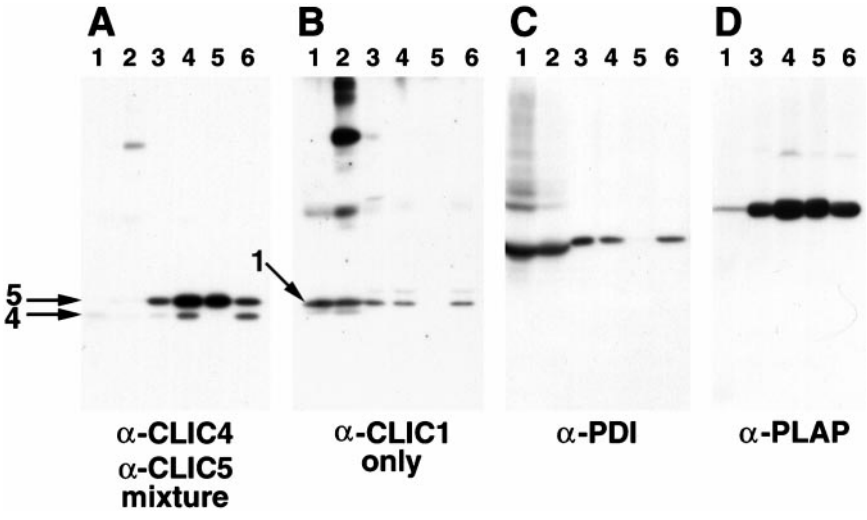

Figure 8. Immunoblot analysis of CLIC proteins in various fractions of human placenta. Approximately equal amounts (7 μg) of total protein from whole placenta (lanes 1), total soluble proteins (lanes 2), placental membranes (lanes 3), and isolated total microvilli (lanes 4) were separated on 12% SDS-PAGE and transferred to membranes. Equal volumes of total (lanes 4), detergent-insoluble (lanes 5), and detergent-soluble (lanes 6) microvillus fractions were also examined. (A) Blot probed with a mixture of antibodies specific for CLIC4 and CLIC5. (B) Blot probed with antibody to CLIC1. (C) Blot probed with a mAb to protein disulfide isomerase (PDI). (D) Blot probed with a mAb to placental alkaline phosphatase (PLAP). The mobilities of the CLIC proteins are indicated by arrows.

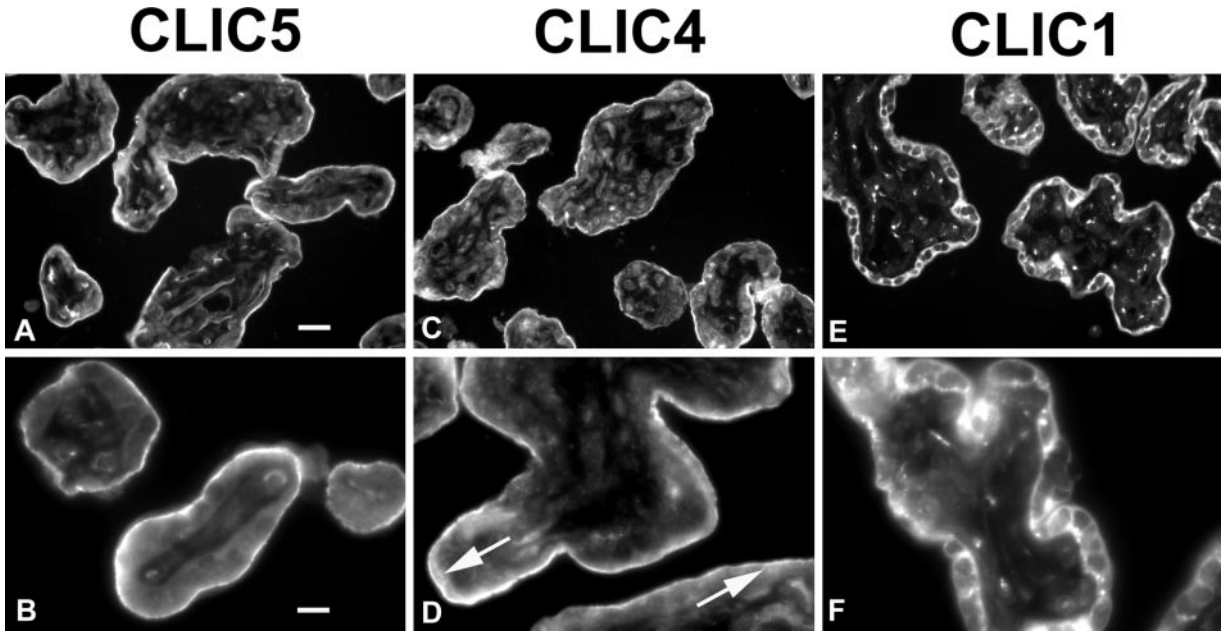

Figure 9. Indirect immunofluorescence localization of CLIC proteins in human placenta. Cryosections were fixed, permeabilized, treated with an antigen-unmasking solution, and stained with primary antibodies specific for CLIC5 (A and B), CLIC4 (C and D), and CLIC1 (E and F). Fluorescein-conjugated anti-rabbit immunoglobulin G was used to detect the primary antibodies. The arrows indicate staining in the apical cytoplasm. The images in the top row were taken with a 16× objective lens (bar in A, 40 μm), and the images in the bottom row were taken with a 40× objective lens (bar in B, 16 μm).

Although it seems reasonable to presume that α-actinin and IQGAP1 help to stabilize and cross-link the bound F-actin in this assay system, the presence of gelsolin is more difficult to explain. One possibility is that gelsolin caps the barbed ends of the short filaments in the assay. The fact that actin and CLIC5 were the most abundant proteins associated with the C terminus of ezrin suggests that CLIC5 interacts directly with either ezrin itself or F-actin. The enhancement of CLIC5 in the presence of added actin suggests that it binds directly to actin. However, the absence of CLIC5 from a cytoskeletal complex trapped in GST control beads incubated with exogenous actin would indicate that the binding of CLIC5 to actin might be enhanced by ezrin, perhaps by the formation of a ternary complex.

Two major lines of evidence indicate that CLIC5 binds to one or more cytoskeletal proteins in placental microvilli. First, despite the 63% identity between CLIC5 and CLIC1, CLIC5 bound with high specificity in the affinity chromatography assay, whereas CLIC1 did not bind at all. Second, biochemical fractionation of placental tissue showed that most of the CLIC5 was associated with the detergent-insoluble cytoskeletal residue of microvilli. In contrast, both CLIC1 and CLIC4 were completely solubilized in cytoskeleton preparations. The dramatic difference in solubility between CLIC5 and CLIC4 is significant given the facts that CLIC4 is 76% identical to CLIC5 and that both proteins are enriched in placental microvilli. Interestingly, CLIC4 displayed weak but specific binding in the affinity chromatography assay, indicating that it might also interact with the cytoskeleton. One explanation for the observed biochemical differences between CLIC4 and CLIC5 is that their binding properties may be regulated by distinct mechanisms. Accordingly, it seems reasonable to predict that bovine p64 might also interact with the actin cytoskeleton, based on the fact that the C-terminal half of this molecule is more closely related to CLIC5 (91% identical) and CLIC4 (77% identical) than to other known members of this gene family.

Tissue Distribution

A direct comparison of CLIC5, CLIC4, and CLIC1 by Northern blot analysis showed major differences in expression among tissues, suggesting that their genes are regulated independently. In general, CLIC1 and CLIC4 appear to be more widely expressed than CLIC5. For example, although CLIC1 and CLIC4 were both detected in liver and pancreas, these tissues expressed little or no CLIC5. CLIC5 was detected at particularly high levels in heart and skeletal muscle, suggesting that this protein plays an important role in muscle cells. We also found that all three family members are expressed in certain tissues, such as kidney and heart. Moreover, although CLIC1 and CLIC4 mRNA appeared to be expressed at higher levels than CLIC5 in placenta, immunofluorescence microscopy revealed that all three proteins are coexpressed in the trophoblast cell.

Subcellular Distribution

Biochemical fractionation and immunofluorescence microscopy showed that CLIC5 and CLIC4 are highly enriched in isolated placental microvilli, suggesting that these proteins function primarily at or near the apical plasma membrane in polarized epithelia. This contradicts most previous studies, which have reported that CLIC proteins reside in intracel-

lular compartments (Duncan *et al.*, 1997; Redhead *et al.*, 1997; Valenzuela *et al.*, 1997; Chuang *et al.*, 1999; Edwards, 1999; Fernandez-Salas *et al.*, 1999; Qian *et al.*, 1999).

Bovine p64, which is most closely related to CLIC5, has been localized to the plasma membrane and to intracellular vesicles in various cell types (Redhead *et al.*, 1992, 1997; Landry *et al.*, 1993). However, recent transfection experiments with the use of p64 mutants suggest that the last six residues of p64 may function to prevent its expression on the plasma membrane of Panc-1 cells (Redhead *et al.*, 1997). This result is intriguing because the last six residues of CLIC5 are identical to those of p64, and we have shown that CLIC5 is enriched at the apical surface of the placental trophoblast. One possibility is that the N-terminal half of p64, which is absent in all the human CLIC family members identified so far, has a dominant influence over the C terminus in determining the subcellular distribution and trafficking of intact p64.

Our results, which show that CLIC4 is concentrated in the apical domain of the placental trophoblast, are consistent with the localization of CLIC4/huH1 in the apical region of renal proximal tubule cells (Edwards, 1999). However, the polarized distribution of CLIC4 in these epithelial cells differs dramatically from what has been found in other cell types. For example, in cells lining the distal portion of the nephron, CLIC4/huH1 is associated with a population of vesicles located in the cytoplasm (Edwards, 1999). Rat brain CLIC4/p64H1 has been localized to the endoplasmic reticulum in transfected cells (Duncan *et al.*, 1997), although recent biochemical and immunoelectron microscopy studies have shown that it is associated with large dense-core vesicles of hippocampal neurons (Chuang *et al.*, 1999). Mouse mc3s5/mtCLIC, which shares 98% overall amino acid identity with rat brain CLIC4/p64H1, has been shown to reside in the cytoplasmic and mitochondrial compartments of mouse keratinocytes and is enriched in mitochondrial fractions of rat liver homogenates (Fernandez-Salas *et al.*, 1999). Together, these findings suggest that the subcellular distribution of CLIC4 may vary according to the specific cell type and functional status of the cell.

In contrast to the polarized distribution of CLIC4 and CLIC5, we found that CLIC1 is distributed throughout the cytoplasm of the placental trophoblast, indicating that CLIC1 is functionally distinct from the other two family members. This hypothesis is supported by our biochemical data, which showed that a substantial portion of CLIC1 in placental extracts remains soluble in the absence of detergent, consistent with results from a previous study in which CLIC1 was found in both soluble and membrane-associated fractions of kidney brush borders (Tulk and Edwards, 1998). In the same study, CLIC1 was found to be concentrated in the brush border of proximal tubule cells with the use of an antibody raised against 13 residues located in the extreme C terminus. However, because 10 of the 13 residues are identical between CLIC1 and CLIC5, it is possible that the staining pattern observed for CLIC1 could actually represent that of CLIC5, especially given that CLIC5 is concentrated in placental microvilli and is expressed at relatively high levels in kidney. Although CLIC1 was originally identified as a nuclear chloride channel protein through a combination of biochemical, immunocytochemical, and electrophysiological studies (Valenzuela *et al.*, 1997), we found that CLIC1 was excluded from the nuclei of the trophoblast epithelium, which exists as a multinucleate syncytium. Clearly, additional localization studies will be required to reconcile the disparate findings on the subcellular distribution of CLIC1 in various cell types.

CONCLUSION

Our findings provide evidence that the cytoskeleton plays a role in the localization and regulation of a novel protein, CLIC5, in polarized epithelial cells. To our knowledge, CLIC4 and CLIC5 are the first chloride transport molecules to be identified as authentic components of the microvilli covering the trophoblast epithelium. Although our immunofluorescence and biochemical data demonstrate that CLIC4 and CLIC5 are enriched in the apical domain of the placental trophoblast, our experiments do not show that they actually reside in the plasma membrane itself. As our comparative analysis suggests, members of the CLIC family, like members of the CLC family of voltage-gated chloride channels (Jentsch *et al.*, 1995), may have different functions in different tissues and cells. One hypothesis is that the CLIC proteins are functionally redundant and that their coexpression ensures that an essential cellular function is fulfilled. Alternatively, these proteins may have related but distinct functions. In support of the latter hypothesis, we have now demonstrated that three highly related proteins, CLIC1, CLIC4, and CLIC5, display dramatic differences in their biochemical properties and subcellular distributions, and that CLIC5 is distinguished by its ability to remain tightly associated with the microvillus cytoskeleton.

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