CLIC-5A is a member of the chloride intracellular channel protein family, which is comprised of six related human genes encoding putative chloride channels. In this study, we found that reconstitution of purified recombinant CLIC-5A into artificial liposomes resulted in a dose-dependent chloride efflux that was sensitive to the chloride channel blocker IAA-94. CLIC-5A was originally isolated as a component of an ezrin-containing cytoskeletal complex from human placental microvilli. Here we show that similar protein complexes can be isolated using either immobilized CLIC-5A or the C-terminal F-actin-binding domain of ezrin and that actin polymerization is required for de novo assembly of these complexes. To investigate the behavior of CLIC-5A in vivo, JEG-3 placental choriocarcinoma cells were stably transfected with epitope-tagged CLIC-5A. In fixed cells, CLIC-5A displayed a polarized distribution and colocalized with ezrin in apical microvilli. Microvillar localization of CLIC-5A was retained after Triton X-100 extraction and was disrupted by treatment with latrunculin B. In transient transfections assays, we mapped a region between residues 20 and 54 of CLIC-5A that is required for targeting of CLIC-5A to microvilli in JEG-3 cells. Interestingly, expression of CLIC-5A in JEG-3 cells did not enhance the rate of iodide efflux in intact cells, suggesting that if CLIC-5A is a chloride channel, its channel activity may be restricted to intracellular membrane compartments in these cells. Regardless of its role in ion transport, CLIC-5A, like ezrin, may play an important role in the assembly or maintenance of F-actin-based structures at the cell cortex.

The CLIC (chloride intracellular channel) proteins are a recently described, closely related family of proteins that are thought to function as chloride channels, although the specific cellular function of any of these proteins remains to be rigorously defined. The human CLIC family is comprised of six known members (CLIC-1–6), all of which share high homology through an approximately 220-amino acid sequence that defines the family (1–7). Sequences N-terminal to the CLIC core sequence vary considerably in length and sequence among the various family members. CLIC proteins show no detectable homology to other families of chloride channels such as cystic fibrosis transmembrane conductance regulator, CLC proteins, or the ligand-gated chloride channels. Enigmatically, the CLIC core region shows weak but significant homology to glutathione S-transferases (8). Unlike most membrane channels, several studies indicate that CLIC proteins are present in cells both as integral membrane proteins and as soluble, apparently cytosolic forms (2, 9, 10). Spontaneous transition from the soluble to the membrane-inserted form has been demonstrated with CLIC-1 and proposed as one potential means of regulating channel activity and subcellular distribution (11, 12). Mechanisms that might regulate membrane insertion remain unknown but could include recruitment to the membrane surface by interactions with membrane phospholipids or by protein-protein interactions with cytoskeletal or integral membrane proteins.

Although several CLICs have been implicated in chloride channel activity using various indirect methods, unambiguous demonstration of channel activity with pure protein has only been demonstrated with CLIC-1 to date. The observations that CLICs are related structurally to glutathione S-transferases and that only a fraction of the CLIC molecules in various cell types is membrane-associated has led to speculations that these molecules may have roles other than ion permeation. The CLIC-5 gene is of particular interest in that its primary RNA transcript is subject to alternative splicing at the first exon. This alternate splicing gives rise to two distinct mRNAs that encode proteins of quite different molecular mass, known as CLIC-5A (251 amino acids) and CLIC-5B (410 amino acids) (6). The C-terminal 238 amino acids of CLIC-5A and -5B are identical. This portion of the molecule contains the CLIC core homology domain. CLIC-5A has an additional 13 amino acids at the N terminus, whereas CLIC-5B has an additional unique 172 N-terminal amino acids. CLIC-5B appears to be the human orthologue of both bovine p64, the 437-amino acid protein that was the first CLIC protein described (13, 14), and avian p62, the CLIC family member that has been implicated in osteoclast bone resorption (15). Both CLIC-5A and CLIC-5B have been shown to be associated with multiprotein complexes containing cytoskeletal and/or scaffolding proteins.

CLIC-5A was initially identified as a component of a cytoskeletal complex containing actin, α-actinin, ezrin, gelsolin, and IQGAP1 from placental microvilli (1). In this paper, we characterize CLIC-5A activity and its interactions with the cytoskeleton in more detail. We demonstrate that, like CLIC-1, recombinant, essentially pure CLIC-5A can be reconstituted in phospholipid membranes to yield a chloride-selective, IAA-94 inhibitable chloride channel activity. We show that immobilized CLIC-5A is capable of recruiting a complex containing actin, ezrin, α-actinin, and an unidentified 70-kDa protein from placental microvillus extracts and explore the ATP and actin polymerization requirements for recruitment of this complex.
Finally, we demonstrate that exogenous epitope-tagged CLIC-5A expressed in JEG-3 choriocarcinoma cells is expressed in an apically polarized pattern where it colocalizes with ezrin in microvilli, a characteristic of CLIC-5A in intact placenta, yet unexpectedly does not enhance plasma membrane anion permeability.

EXPERIMENTAL PROCEDURES

Materials—Placental microvilli were isolated from fresh tissue according to published procedures (16). Affinity-purified antibodies that specifically recognize CLIC-5A and CLIC-4 (1), ezrin (17), and α-actinin (18) have been described. Mouse monoclonal antibodies against actin (clone AC-40), gelsolin (clone 2), and Xeps epoxide were purchased from Sigma, Transduction Laboratories (Lexington, KY), and Invitrogen, respectively. Peroxidase-labeled secondary reagents were obtained from Sigma. Species-specific secondary antibodies conjugated to Alexa-488 or Texas Red, rhodamine-phalloidin, and Prolong antifade medium were from Molecular Probes (Eugene, OR).

Fusion Proteins—A GST (19) fusion protein containing the F-actin-bind- ing site of ezrin (residues 556–586) has been described (19). To generate full-length (residues 1–251) CLIC-5A fusion proteins, a previously charac- terized cDNA (1) was amplified by PCR using a forward primer with a BamHI site at the 5’ end. The product was ligated into a TA cloning vector (Invitrogen), digested with BamHI, and then subcloned into pGEX-2T (Amersham Biosciences) and pcDNAs3.1Hic/H (Invitrogen) for expression of GST and Xeps-tagged fusion proteins, respectively. A similar strategy was employed to make various truncated forms of CLIC-5A utilizing the pcDNA3.1Hic/H vector. GST fusion proteins were expressed in Escherichia coli strain BL21 and purified as described previously (19). Plasmids encoding Xeps-tagged CLIC-5A were prop- agated in the DH5α strain of E. coli.

Preparation of CLIC-5A for Reconstitution—Initial steps in purifica- tion of CLIC-5A were identical to those described previously for CLIC-5A (11) except that 10 mM 2-mercaptoethanol was substituted for dithio- reitol in all steps up to and including thrombin digestion and that the 40,000 rpm centrifugation step prior to loading the glutathione column was omitted. CLIC-5A was released from the fusion protein by digestion with thrombin as described previously (11, 20). Those proteins not re- leased from the column by digestion with thrombin were subse- quently eluted with 20 mM reduced glutathione, 10 mM 2-mercaptoeth- anol, 10 mM Tris, pH 8.0, and 1.4% n-octylglucopyranoside. The fraction released from the glutathione column by digestion with thrombin was diluted 3-fold with Buffer A (5 mM dithiothreitol, 10 mM Tris, pH 8.0, and 1.4% n-octylglucopyranoside), loaded onto a 1 mM High-Performance liquid chromatography cartridge Bio-Rad that had been equilibrated in Buffer A, washed with 20 mM of Buffer A, and eluted with a linear gradient of 0–0.5 M NaCl in Buffer A. Samples containing CLIC-5A were pooled and applied to a Sephacryl S1000 column equilibrated with Buffer A. The peak CLIC-5A fractions were pooled. To concentrate CLIC-5A, pooled fractions were applied to a 1-mL High-Q anion exchange cartridge that had been equilibrated with 0.1% SDS, 1% Triton X-100, 10 mM 2-mercaptoethanol, 2.5 mM EGTA, 5 mM MgCl2, and 0.5 mM benzamidine. The extracts were diluted at 10 mM in 20 mM Tris, pH 7.4, 1 µM leupeptin, 1 µg/ml pepstatin A, 1 mM phenylmethylsulfonyl fluoride, 0.5 mM benzamidine. The extracts were centrifuged at 100,000 × g for 15 min to pellet insoluble material. The supernatants were mixed with 20 µl of protein A-Sepharose beads (Sigma) and either 6 µl of CLIC-5A antiserum (B132) or the correspond- ing preimmune serum. After 3 h, the beads were washed extensively with RIPA, and the immune complexes were eluted by boiling in Laemmli sample buffer.

Cell Fractionation—Triton cytoskeletons were prepared essentially as previously described (22). Briefly, the cells were rinsed with PBS and then incubated for 5 min at room temperature in the presence or absence of 0.5% Triton X-100 in 10 mM HEPES, pH 7.4, 100 mM NaCl, 14 mM 2-mercaptoethanol, 2.5 mM EDTA, 5 mM MgCl2, 1 mM phenylmethylsulfonyl fluoride, and 0.5 mM benzamidine. The detergent-solu- ble material was removed by boiling in Laemmli sample buffer after rinsing with PBS, the cells were either fixed with ice-cold methanol and processed for immunofluorescence microscopy or solubilized in Laemmli sample buffer for immunoblot analyses.

Iodide Efflux Assay—JEG-XC5–1 and JEG-XC5–3 cells were grown in triplicate cultures in 24-well plates. The cells were loaded with 50 mCi (131I) for 20 min and centrifuged at 10,000 rpm for 30 min to pellet insoluble material. The supernatants were mixed with 20 µl of protein A-Sepharose beads (Sigma) and 6 µl of CLIC-5A antiserum (B132) or the corresponding preimmune serum. After 3 h, the beads were washed extensively with RIPA, and the immune complexes were eluted by boiling in Laemmli sample buffer.

RESULTS

Purified CLIC-5A Mediates Chloride Conductance in Artificial Liposomes—GST-CLIC-5A fusion protein was expressed in bacteria, and a cleared lysate was prepared (Fig. 1, lane 1). The lysate was allowed to bind to glutathione-agarose, washed ex-
tensively, and then digested with thrombin while still bound to the resin, releasing CLIC-5A with an apparent molecular mass of about 32 kDAs on SDS page (Fig. 1, lane 3). The resin was then eluted with reduced glutathione, releasing the 27-kDa GST portion of the fusion protein along with some residual undigested 60-kDa fusion protein (Fig. 1, lane 2). The material in lane 3 was further purified using ion exchange chromatography over a Mono-Q column (lane 4), then purified by gel filtration over a Sephacryl S-100 column (lane 5), and finally concentrated by passage over Mono-Q column again (lane 6).

No other proteins were apparent in the final product by Coomassie Blue staining on overloaded SDS-PAGE gels. Interestingly, CLIC-5A eluted from the Sephacryl S-100 column earlier than expected for a protein predicted to be 28 kDa. To examine the apparent molecular mass in more detail, purified CLIC-5A was passed over a Sephacryl S-300 column that had been calibrated using commercially available molecular mass standards. CLIC-5A eluted as a single species with an apparent molecular mass of 75 kDa, suggesting that this purified CLIC-5A is present in solution as a dimer (data not shown).

Purified recombinant CLIC-5A was reconstituted into phospholipid vesicles and assayed for chloride-selective permeability using a chloride-selective electrode based system as previously described (11). Preparations of CLIC-5A reproducibly yielded increased chloride permeability compared with control vesicles reconstituted in the absence of protein (Fig. 2). The results in Fig. 2A demonstrate that the rates of chloride efflux increased with increasing amounts of CLIC-5A in the vesicles. The absolute rates of chloride efflux were: 0 μg/ml CLIC-5A, 0.71 ± 0.16%/s; 50 μg/ml CLIC-5A, 1.33 ± 0.31%/s; 100 μg/ml CLIC-5A, 1.68 ± 0.062%/s; 200 μg/ml CLIC-5A, 2.53 ± 0.38%/s (n = 3 for each data point).

IAA-94 is an inhibitor that was used to purify the founding member of the CLIC family, p64 (13). IAA-94 has been shown to inhibit CLIC-1 in an identical assay system with an apparent IC₅₀ of 9 μM (11). To determine whether IAA-94 inhibits channel activity of recombinant CLIC-5A, vesicles were prepared in the absence of protein or with 200 μg/ml CLIC-5A. The vesicles were then assayed for chloride-selective efflux in the presence or absence of 50 μM IAA-94 (Fig. 2B). To allow comparison between different preparations, the results were normalized to control rates. Normalized rates of chloride efflux were: control (no protein) vesicles, 1.00 ± 0.015%/s (n = 2); CLIC-5A vesicles, 2.44 ± 0.11%/s (n = 3); CLIC-5A plus IAA-94 vesicles, 1.26 ± 0.32%/s (n = 3). The chloride efflux rate from the CLIC-5A vesicles was significantly greater than the control vesicles or the CLIC-5A plus IAA-94 vesicles (p < 0.03).

To determine whether the substance responsible for the CLIC-5A-associated chloride efflux is heat-sensitive, 200 μg of untreated or heat-denatured CLIC-5A was reconstituted into phospholipid vesicles in a 1-ml volume. The resulting vesicles were assayed for chloride-selective permeability (Fig. 2C). Normalized rates of chloride efflux were: control (no protein) vesicles, 1.00 ± 0.18%/s (n = 2); CLIC-5A vesicles, 2.69 ± 0.48%/s (n = 3); boiled CLIC-5A vesicles, 1.53 ± 0.11%/s (n = 3). The efflux rate from CLIC-5A vesicles was significantly greater than either the control vesicles or the vesicles reconstituted with heat-denatured CLIC-5A (p < 0.05).

CLIC-5A Promotes the Assembly of an F-actin-containing Complex in Vitro—CLIC-5A was originally identified as a cytoskeletal-associated protein in human placental microvilli. In these original studies, a GST fusion protein containing the C-terminal F-actin-binding site of ezrin (GST-ez/556–586) was incubated with soluble microvillus extracts in the presence of ATP, resulting in the isolation of a protein complex containing actin, α-actinin, ezrin, gelsolin, IQGAP1, and CLIC-5A (1). In this study, the same pull-down assay was used, but in addition, a GST fusion protein containing full-length CLIC-5A was also included to test whether CLIC-5A itself could bind cytoskeletal proteins. As shown in Fig. 3, actin, α-actinin, ezrin, and IQGAP1 showed specific, ATP-dependent binding to GST-CLIC-5A as well as GST-ez/556–586. Although CLIC-5A essentially mimicked the cytoskeletal binding activity of GST-ez/
CLIC-5A Concentrates in the Microvilli of JEG-3 Choriocarcinoma Cells—To further investigate the function of CLIC-5A, we generated a stably transfected cell line expressing CLIC-5A as a His6-tagged fusion protein that also contained an Xpress epitope tag at its N terminus. Human JEG-3 choriocarcinoma cells were chosen as a host, because these epithelial cells share several morphological and biochemical attributes with the intact human placental trophoblast. Although JEG-3 cells express CLIC-4 (24), immunoblot analysis of total JEG-3 extracts revealed that CLIC-5A was undetectable in these cells (data not shown; for RIPA soluble extracts; Fig. 5). From a pool of stably transfected cells, two clones, designated JEG-XC5–1 and JEG-XC5–3, were selected for analysis. Immunoprecipitates were prepared using an antibody raised against CLIC-5A (1) followed by immunoblotting. A ~40-kDa band, corresponding to the expected mobility of Xpress-tagged CLIC-5A, was readily detected in CLIC-5A immunoprecipitates from JEG-XC5–1 cells but not JEG-XC5–3 cells (Fig. 5) or untransfected JEG-3 cells (data not shown). Because JEG-XC5–3 cells grew well in the presence of selective antibiotic but failed to produce detectable levels of CLIC-5A, these cells, together with untransfected JEG-3 cells, were used as negative controls in subsequent experiments. Neither ezrin nor CLIC-4 was detectable in CLIC-5A immunoprecipitates prepared from JEG-XC5–1 cells (data not shown).

The subcellular distribution of CLIC-5A was examined by immunofluorescence microscopy of fixed JEG-3 cells stained with ezrin (Fig. 6), a marker for apical microvilli in these and a variety of other epithelial cell types (25, 26). CLIC-5A was mainly concentrated in the apical region of JEG-XC5–1 cells, where it colocalized with ezrin in microvilli (Fig. 6, A–H). The CLIC-5A staining was specific to JEG-XC5–1 cells, because background staining was negligible in parallel samples of JEG-XC5–3 cells (Fig. 6, I–L) or untransfected JEG-3 cells (data not shown). The colocalization of CLIC-5A and ezrin in microvilli was confirmed using confocal microscopy in both the X–Y and X–Z planes (see Fig. 9). It is noteworthy that in addition to the microvilli themselves, distinct puncta were observed in regions between microvilli and in close proximity to the microvilli. In many cases, ezrin staining was not detectable in these nonmi-

556–586, there were several notable differences. The GST-ez/556–586 bound lower quantities and a higher ratio of actin: α-actinin than GST-CLIC-5A. In addition, gelsolin and endogenous CLIC-5A were readily detectable in GST-ez/556–586 eluates but not in GST-CLIC-5A eluates, indicating that CLIC-5A does not interact directly with gelsolin or itself in this assay. Interestingly, a band migrating at ~70 kDa (p70) was observed in eluates from both GST-CLIC-5A and GST-ez/556–586 beads. Increased amounts of p70 binding to GST-ez/556–586 beads were observed when ATP was included, whereas similar amounts of p70 bound to GST-CLIC-5A beads regardless of inclusion of ATP. Although alkaline phosphatase is a major 68-kDa protein in isolated placental microvilli (23), immunoblot analysis indicated that p70 is distinct from alkaline phosphatase (data not shown).

Although it is possible that preformed cytoskeletal complexes present in soluble microvillus extracts might bind to GST-CLIC-5A or GST-ez/556–586, the fact that cytoskeletal protein binding is enhanced in the presence of ATP indicated that actin polymerization might facilitate de novo assembly of the cytoskeletal complexes. To distinguish between these two possibilities, affinity beads were incubated with microvillus extracts and ATP in the absence or presence of latrunculin B (LatB), a drug that disrupts actin polymerization. As shown in Fig. 4, the addition of LatB dramatically reduced the levels of actin and α-actinin binding to GST-CLIC-5A as well as GST-ez/556–586. It is noteworthy that binding of endogenous CLIC-5A to GST-ez/556–586 was diminished in the presence of the drug, suggesting that CLIC-5A does not interact directly with this C-terminal fragment of ezrin. In contrast, LatB had little effect on the binding of endogenous ezrin or p70 to GST-CLIC-5A, suggesting that either or both of these proteins could bind directly to CLIC-5A.

Fig. 3. Isolation of cytoskeletal proteins from placental microvillus extracts in a pull-down assay using immobilized CLIC-5A and the C-terminal 30 residues of ezrin. Glutathione-agarose beads containing equimolar amounts of GST alone as a control, GST-CLIC-5A, or GST-ez/556–586 were incubated with buffer alone or microvillus extracts in the presence or absence of ATP. After washing, the bound proteins were eluted by boiling in Laemmli sample buffer and run on 12% SDS-PAGE. A, gel stained with Coomassie Blue. The white asterisks indicate fusion proteins; the black asterisk indicates actin. B, immunoblots of samples corresponding to lanes in A probed with various antibodies against cytoskeletal proteins.

Fig. 4. Effect of LatB on isolation of cytoskeletal proteins from placental microvillus extracts. Glutathione-agarose beads containing equimolar amounts of GST, GST-CLIC-5A, or GST-ez/556–586 were incubated with buffer alone or microvillus extracts supplemented with ATP in the presence or absence of 10 μM LatB. The samples were processed as described in the legend to Fig. 1. A, Coomassie Blue-stained gel. B, corresponding immunoblot.
crovillar CLIC-5A-containing puncta, consistent with the possibility that some CLIC-5A associates with a population of vesicles beneath the plasma membrane. CLIC-5A was not detected in F-actin-containing stress fibers or focal contacts in cells stained with rhodamine-phalloidin (data not shown). In addition, no differences were apparent between JEG-XC5−1 and JEG-XC5−3 cells in terms of overall pattern of F-actin or ezrin staining, length of microvilli, or density of microvilli.

Because LatB had a major effect on the association of cytoskeletal proteins with CLIC-5A and ez/556−586 in the in vitro pull-down assay (Fig. 4), we examined the effects of this drug on the distribution of CLIC-5A and ezrin in JEG-XC5−1 cells (Fig. 7). LatB induced dramatic changes in cell morphology within 15 min of drug treatment, including the formation of numerous retraction fibers and a variable increase in cell height (data not shown). In contrast to the numerous microvilli covering the surface of control cells treated with Me2SO alone, cells treated with LatB displayed relatively few microvilli and bright patches of CLIC-5A and ezrin at the apical surface. Interestingly, CLIC-5A and ezrin showed a remarkable colocalization in these apical patches (Fig. 7, E−H, arrows).

To further test properties of the observed association between CLIC-5A and ezrin, the effect of nonionic detergent extraction on solubility of CLIC-5A was assessed. Treatment of cells with nonionic detergents such as Triton X-100 is known to solubilize typical cytoplasmic and membrane proteins while not solubilizing the F-actin cytoskeleton and its associated proteins. Therefore, we extracted cultures of JEG-XC5−1 cells with Triton X-100 and examined the distribution of CLIC-5A and ezrin by immunofluorescence microscopy and immunoblotting. As shown in Fig. 8A, CLIC-5A and ezrin staining were readily detected in the apical microvilli of methanol-fixed cells regardless of pretreatment with detergent. However, there was a reduction in staining intensity for both proteins and a corresponding increase in the contrast of the microvillar structures compared with untreated control cells. This suggests that CLIC-5A and ezrin were selectively retained by actin-rich microvilli and preferentially extracted in nonmicrovillar regions (Fig. 8A, compare panels D and H). Accordingly, immunoblot analysis revealed that a substantial amount of CLIC-5A and ezrin was retained in the detergent-insoluble cytoskeletal fraction, although not surprisingly, most CLIC-5A and ezrin was solubilized during the detergent extraction procedure (Fig. 8B).

**Targeting of CLIC-5A to Apical Microvilli in JEG-3 Cells—**
To determine which regions of CLIC-5A are required for its accumulation in the apical microvilli of JEG-3 cells, we examined the localization of various Xpress-tagged CLIC-5A con-
constructs with deletions of the N and C termini. Transiently transfected JEG-3 cells were fixed and then double-labeled with antibodies against the Xpress epitope and ezrin. The data are summarized in Table I, and representative confocal images are shown in Fig. 9. Constructs in which the N-terminal 13 or 19 amino acids were deleted were expressed in a pattern indistinguishable from that of the full-length CLIC-5A and showed clear colocalization with ezrin in the apical microvilli. N-terminal deletions to position 53 or beyond eliminated the colocalization with ezrin. Although the N-terminal deletion containing residues 54–251 and the C-terminal deletion containing residues 1–220 are localized mainly in the apical region of the cells, there is little, if any, colocalization with ezrin in microvilli.

**TABLE I**

Summary of effects of N- and C-terminal CLIC-5A deletions on targeting to microvilli in JEG-3 cells

| CLIC-5A residues | Microvillar localization |
|------------------|--------------------------|
| Full length (control) | 1–251 | + |
| N-terminal deletions | 14–251 | + |
| | 20–251 | + |
| | 54–251 | – |
| | 108–251 | – |
| | 160–251 | – |
| C-terminal deletions | 1–220 | – |
| | 1–168 | – |
| | 1–115 | – |

**FIG. 8. Effect of Triton X-100 on solubility of CLIC-5A in JEG-XC5–1 transfectants.** A, cells were treated for 5 min in buffer containing 0.5% Triton X-100 (panels E–I) or not (panels A–D), as described under “Experimental Procedures.” After washing in PBS, the cells were fixed in methanol and stained with Xpress monoclonal antibody to detect CLIC-5A (red), affinity-purified ezrin antibody (green), and DAPI (blue). Superimposed images are shown (MERGE). Bar, 10 μm. B, cells were processed as described above, either in the absence (lanes 1 and 2) or presence (lanes 3 and 4) of 0.5% Triton X-100 in the buffer. After collecting the buffer and rinsing with PBS, the cells were scraped in Laemmli sample buffer (I). Immunoblot containing equal volumes of detergent-soluble (S) and detergent-insoluble (I) fractions was probed with antibody against the Xpress epitope and ezrin.

**FIG. 9. Localization of N- and C-terminal deletion mutants of CLIC-5A in microvilli of JEG-3 cells.** JEG-3 cells were transiently transfected with Xpress-tagged constructs of CLIC-5A, fixed, permeabilized, and then stained with antibodies against the Xpress epitope (red) and ezrin (green). Counterstaining with DAPI (blue) is only shown in the superimposed images (MERGE). Optical sections (1 μm thick) were taken at 0.5-μm intervals on a confocal microscope and processed as X-Y projections and corresponding X-Z slices. Full-length CLIC-5A residues 1–251, as well as the N-terminal deletions containing residues 14–251 and 20–251 colocalize well with ezrin in apical microvilli. Although the N-terminal deletion containing residues 54–251 and the C-terminal deletion containing residues 1–220 are localized mainly in the apical region of the cells, there is little, if any, colocalization with ezrin in microvilli. Bars, 10 μm.

Association of CLIC-5A with the Plasma Membrane—Previous studies on CLIC-1 and CLIC-4, which are believed to span the membrane at least once, have indicated that the N terminus is on the external surface of the membrane, whereas the C terminus is cytoplasmic (2, 9, 10). To determine whether the Xpress epitope located at the N terminus of CLIC-5A is exposed to the exterior surface of the plasma membrane, permeabilized and nonpermeabilized JEG-XC5–1 cells were stained with antibody against the Xpress epitope. To assess the membrane permeability of individual cells, specimens were double-labeled with rhodamine-phalloidin. Truly nonpermeabilized cells, identifiable by the absence of rhodamine-phalloidin staining, did not exhibit any specific CLIC-5A staining (data not shown).

Thus, the Xpress epitope is only accessible to the antibody if membranes are permeabilized, suggesting that the N terminus of CLIC-5A is not exposed to the exterior surface of JEG-3 cells.

**Effect of CLIC-5A on Anion Efflux in Vivo**—To test the possibility that CLIC-5A might function as a chloride channel in vivo, we compared the ability of JEG-XC5–1 and JEG-XC5–3 control cells to mediate bulk anion transport utilizing a conventional iodide efflux assay. Surprisingly, no differences in the average rates of iodide efflux were observed between the

sequences near both the N terminus (between positions 20 and 54) and the C terminus (beyond position 220) are necessary but not sufficient for microvillar targeting of CLIC-5A.
two cell lines (data not shown). Similar results were obtained after stimulation of cells with forskolin, phorbol ester, or LatB.

DISCUSSION

The CLIC-5A protein was originally identified as a protein recruited from extracts of purified placental microvilli by ezrin (1), a membrane-cytoskeletal linking protein that is highly enriched in placental syncytiotrophoblast epithelium (16). Although CLIC-5A was shown to colocalize with ezrin in the apical domain of placental epithelium, the physiologic roles of CLIC-5A in the placenta and placental cytokeratin remain unknown. An attractive hypothesis has been that cytoskeletal association could facilitate or regulate channel insertion into microvilli as needed, leading to a cytoskeletal regulated plasma membrane chloride channel. This paper addresses these possibilities. Our experiments show that 1) purified recombinant CLIC-5A has chloride channel activity in vitro, 2) immobilized CLIC-5A recruits a cytoskeletal complex from placental microvilli extracts, 3) expression of epitope-tagged CLIC-5A in a choriocarcinoma cell line results in colocalization of CLIC-5A with ezrin in an apical, microvilli-associated distribution that is resistant to detergent extraction, and 4) a region between residues 20 and 54 is necessary but not sufficient for targeting of CLIC-5A to microvilli. However, we do not find increased plasma membrane anion permeability in cells stably expressing CLIC-5A. These novel observations have important ramifications for our understanding of CLIC family proteins.

The behavior of CLIC-5A, in terms of channel activity, was comparable with results obtained previously for CLIC-1 (11). CLIC-5A channel activity varied with the amount of CLIC-5A used in reconstitution and the relationship between mass of protein reconstituted and fractional chloride efflux rate is roughly similar to that of CLIC-1. Therefore, the specific activity for chloride channel activity as measured by this assay is similar between CLIC-1 and CLIC-5A. IAA-94 is a chloride channel inhibitor that has been shown to inhibit CLIC-1 with an IC₅₀ of about 10 μM in the chloride efflux assay (20). Here we found that 50 μM IAA-94 inhibited the CLIC-5A activity nearly completely, indicating that CLIC-5A is at least as sensitive to the drug as is CLIC-1. Finally, the CLIC-5A-associated chloride efflux activity is sensitive to heat denaturation, supporting the interpretation that the channel activity is in fact caused by the purified CLIC-5A protein rather than a nonprotein contaminant. These results conclusively demonstrate that CLIC-5A can function as a chloride-selective channel in vitro. Although the chloride efflux assay used here, which employs a population of vesicles, cannot yield single channel properties, it nonetheless provides the first evidence that CLIC-5A meets the biochemical definition of an anion channel.

The in vitro pull-down experiments revealed that CLIC-5A does indeed interact with components of the cortical actin cytoskeleton, confirming results that originally led to its identification (1). In this study, we demonstrated that immobilized CLIC-5A can recruit a cytoskeletal complex from placental microvillus extracts that is similar but not identical to that recruited by the C-terminal tail of ezrin. Like ezrin, immobilized CLIC-5A can promote de novo assembly of a complex containing actin, α-actinin, ezrin, and p70 in a process that involves actin polymerization. Binding assays carried out in the absence of ATP or in the presence of LatB, which blocks actin polymerization, indicate that the interaction with actin and α-actinin require actin polymerization. It is unlikely that CLIC-5A interacts directly with F-actin, because the levels of actin binding to CLIC-5A were much lower than that for the C-terminal F-actin-binding domain of ezrin. This interpretation is consistent with results from brain extracts showing that CLIC-4 associates with an actin-containing protein complex but does not co sediment with purified F-actin isoforms in spin down assays (27). Although our data indicate that CLIC-5A does not bind directly to the C terminus of ezrin, it is likely that CLIC-5A interacts directly with another region of the ezrin protein. Consistent with this possibility, the binding of endogenous ezrin to CLIC-5A was largely unaffected by ATP and LatB. Similarly, ATP and LatB had little effect on the binding of p70, indicating that this binding is independent of actin polymerization and that p70 may interact directly with the immobilized CLIC-5A. We postulate that immobilized CLIC-5A binds to an accessory protein, such as p70 or ezrin, which then recruits F-actin and other actin-binding proteins. In contrast, immobilized ezrin C terminus binds directly to F-actin, resulting in recruitment of actin-binding proteins and, in turn, CLIC-5A.

Our in vivo data further establish that CLIC-5A associates with the cortical actin cytoskeleton. In addition to the polarized distribution of CLIC-5A and its colocalization with ezrin in the apical microvilli of transfected JEG-3 cells, we found that a substantial fraction of CLIC-5A is resistant to extraction with nonionic detergent and that the localization of CLIC-5A is dramatically altered by disruption of the actin cytoskeleton. There is increasing evidence that other CLIC proteins can associate with the cytoskeletal proteins and scaffolding complexes. Recently, Griffon et al. (28) have shown that CLIC-6 is part of a submembranous complex consisting of dopamine D₂-like receptors, multipDZ protein, and radixin, a close relative of ezrin. In addition, Shanks et al. (6) have demonstrated that multiple CLIC proteins, including CLIC-1, CLIC-4, CLIC-5A, CLIC-5B, and parchorin, can bind directly to AKAP350, a scaffolding protein that sequesters several signaling enzymes in the Golgi-centrosome region of a variety of cell types, including JEG-3 cells (24, 29). Shanks et al. (6) also showed a green fluorescent protein fusion protein containing residues 178–410 of CLIC-5B, which are identical to residues 20–251 of CLIC-5A, colocalizes with AKAP350 in the Golgi-centrosome region of HCA-7 colon carcinoma cells. Here, these exact same residues resulted in targeting to microvilli in our cell system, suggesting that subcellular targeting of CLIC-5 splice variants is differentially regulated in a cell type-specific manner. More recently, studies using a Caenorhabditis elegans CLIC have shown that the N-terminal 55 amino acids are adequate to confer correct subcellular localization in the excretory system of the worm, consistent with our findings (30).

We performed iodide efflux experiments to determine whether JEG-3 cells expressing exogenous CLIC-5A have increased plasma membrane anion permeability and found to our surprise that they do not. The are many potential explanations for this observation, but three of the most salient are 1) that (as suggested by the failure to detect exogenous CLIC-5A in nonpermeabilized cells) the protein is excluded from the plasma membrane, 2) that we failed to use the correct stimulus to activate channel activity, or 3) that its primary function in these cells is not as a channel but as a cytoskeletal component. Further experiments will be needed to determine whether any of these possibilities are correct.

In this report, we have demonstrated that CLIC-5A has the capacity to function as a chloride channel in vitro and that it associates with the cortical actin cytoskeleton in vitro and in vivo. Unexpectedly, however, CLIC-5A did not influence the rate of chloride efflux in intact cells, raising the intriguing possibility that interaction of CLIC-5A with the cytoskeleton may preclude channel activity or insertion of CLIC-5A into the plasma membrane. Whether the cytoskeleton influences CLIC-5A function or whether CLIC-5A influences cytoskeletal dynamics remains to be determined.

Acknowledgments—We are indebted to Anthony Bretscher (Cornell University) for providing affinity-purified antibodies against ezrin and...
α-actinin. We thank Darlene Berryman (Ohio University) for continued encouragement, advice, and critical reading of the manuscript.

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J. Biol. Chem. 2004, 279:34794-34801.
doi: 10.1074/jbc.M402835200 originally published online June 7, 2004

Access the most updated version of this article at doi: 10.1074/jbc.M402835200

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