AKAP350 at the Golgi Apparatus

II. ASSOCIATION OF AKAP350 WITH A NOVEL CHLORIDE INTRACELLULAR CHANNEL (CLIC) FAMILY MEMBER*

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AKAP350 can scaffold a number of protein kinases and phosphatases at the centrosome and the Golgi apparatus. We performed a yeast two-hybrid screen of a rabbit parietal cell library with a 3.2-kb segment of AKAP350 (nucleotides 3611–6813). This screen yielded a full-length clone of rabbit chloride intracellular channel 1 (CLIC1). CLIC1 belongs to a family of proteins, all of which contain a high degree of homology in their carboxyl termini. All CLIC family members were able to bind a 133-amino acid domain within AKAP350 through the last 120 amino acids in the conserved CLIC carboxyl termini. Antibodies developed against a bovine CLIC, p64, immunoprecipitated AKAP350 from HCA-7 colonic adenocarcinoma cell extracts. Antibodies against CLIC proteins recognized at least five CLIC species including a novel 46-kDa CLIC protein. We isolated the human homologue of bovine p64, CLIC5B, from HCA-7 cell cDNA. A splice variant of CLIC5, the predicted molecular mass of CLIC5B corresponds to the molecular mass of the 46-kDa CLIC immunoreactive protein in HCA-7 cells. Antibodies against CLIC5B colocalized with AKAP350 at the Golgi apparatus with minor staining of the centrosomes. AKAP350 and CLIC5B association with Golgi elements was lost following brefeldin A treatment. Furthermore, GFP-CLIC5B-(178–410) targeted to the Golgi apparatus in HCA-7 cells. The results suggest that AKAP350 associates with CLIC proteins and specifically that CLIC5B interacts with AKAP350 at the Golgi apparatus in HCA-7 cells.

Cells contain a dynamic organization of signaling molecules and proteins required for the transduction of intracellular signaling events. Thus, cellular organization is crucial for intracellular signals to function correctly. The study of how the cell completes this task has revealed molecular scaffolds that allow spatial control of signaling events (1, 2). The study of signaling molecules scaffolded in specific locations within the cell has led to an expanded understanding of the cellular functions that they control.

Scaffolding proteins are utilized in the cell to localize signaling proteins to specific areas facilitating precise cellular function. Protein kinase A-anchoring proteins (AKAPs) are one class of scaffolding protein utilized by the cell to anchor the regulatory subunit of type II CAMP-dependent protein kinase A (3) to specific locations within the cell (4). In addition to the targeting of anchored protein kinase A, another key function of AKAPs is the recruitment of other signaling proteins (5). Often an AKAP will scaffold both kinases and phosphatases, thereby coordinating specific signaling events (6). Thus, protein scaffolds can assemble protein kinase and phosphatase systems in close proximity of their intended substrates.

We have previously defined one AKAP, AKAP350, which is the product of a multiply spliced gene family and is localized at the centrosome (7). This protein has also been observed on the Golgi apparatus, where it was identified as the centrosome and Golgi apparatus-localized protein kinase N-associated protein (CG-NAP) (8). The sequence of CG-NAP is identical to AKAP350 designated according to its 450-kDa size (9), but we will refer to AKAP350/CG-NAP/AKAP450 as AKAP350 based on the original identification of this protein by Keryer et al. (10) as a 350-kDa protein kinase A-anchoring protein at the centrosome.

The function of a particular AKAP is defined by its localization and the proteins bound to the scaffold (10). AKAP350 has putative binding sites for protein phosphatase 1, protein kinase N, protein phosphatase 2A (8), protein kinase Ce (11), and phosphodiesterase 4D3 (12) as well as two protein kinase A binding sites (7, 8). However, previous studies have not identified proteins other than kinases and phosphatases interacting with AKAP350. We hypothesized that AKAP350 also bound to other signaling proteins based on the large size of the protein. Using yeast two-hybrid screening we now show that the chloride intracellular channel (CLIC) family of proteins interacts with a region of AKAP350 previously denoted as the peri-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF083037 and AY032602.

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The abbreviations used are: AKAP, protein kinase A-anchoring protein; CLIC, chloride intracellular channel; PHR, pericentrin homology region; PBS, phosphate-buffered saline; TBS, Tris-buffered saline.
identified previous (15). Full-length human CLIC1, CLIC4, CLIC5A, and the PHR region of AKAP350 (nucleotides 3611–40974) were cloned into the binding domain vector pBDGal-4-Cam (Stratagene) with the use of EcoRI and SalI sites. The plasmids harboring the HIS3 and β-galactosidase reporter genes were used to screen ~1 million clones as described previously (15). The isolated clone was rescued into XL-1 Blue bacteria, and plasmid DNA was prepared using Qiagen Miniprep kits. All DNA sequencing was performed using dye terminator chemistry automated sequencing in the Molecular Biology Core Facility at the Medical College of Georgia. The isolated clone and the “bait” pBDGal-4-Cam vector both gave negative results when analyzed by themselves in yeast two-hybrid assays.

**EXPERIMENTAL PROCEDURES**

**Materials—Oligonucleotides** were synthesized by the Medical College of Georgia Molecular Biology Core Facility. Cy3-conjugated species-specific secondary antibodies were purchased from Jackson Immunoresearch Laboratories (West Grove, PA). Prolong Antifade and Alexa 488-conjugated species-specific secondary antibodies were from Molecular Probes, Inc. (Eugene, OR). [α-32P]dCTP was purchased from PerkinElmer Life Sciences.

**Yeast Two-hybrid Screening**—A rabbit parietal cell two-hybrid library in pAD-GAL4 (15) was screened with 3.2 kb of AKAP350 sequence (nucleotides 3611–6813, GenBank™ accession number AF083037) cloned into the binding domain vector pBDGal-4-Cam (Stratagene). The use of EcoRI and SalI sites and being tested for interaction in the yeast two-hybrid assay as described previously (15). Full-length human CLIC1, CLIC4, CLIC5A, and parochin constructs (14, 16) were cloned into pADGal-4 and tested for interaction with the AKAP350-PHR-pBDGal-4-Cam bait in the yeast two hybrid assay as described previously (15). Positive results were recorded if β-galactosidase assay was positive within 3 h. The pADGal-4 vectors produced negative results when analyzed by alone.

**Antibodies**—The 14G2 monoclonal anti-AKAP50 antibody was developed as described previously (7). The 121, 656, and B132 polyclonal anti-CLIC antibodies were prepared as described previously (14, 17). The specific polyclonal anti-CLIC5B antibody was raised against the peptide containing amino acids 14–26 (QNERTYEVPDPEE) of the decapeptide (GenBank™ accession number U93205). Subsequent yeast two-hybrid binary assays were performed with 10 μg/ml brefeldin A for 1 h at 37 °C. Cells were washed in phosphate-buffered saline and fixed with 4% paraformaldehyde for 15 min at room temperature. Cells were blocked with 17% donkey serum, 0.3% Triton-X100 in phosphate-buffered saline and then incubated sequentially with 14G2 (mouse anti-AKAP350) (1:85), mouse anti-p58 (1:85) and the appropriate secondary antibodies. Confocal images were captured with a Zeiss microscope.

**Cellular Fractionation**—HCA-7 cells were grown on glass coverslips to confluence. The cells were then incubated in the absence or presence of 10 μg/ml brefeldin A for 1 h at 37 °C. Cells were washed in phosphate-buffered saline and fixed with 4% paraformaldehyde for 15 min at room temperature. Cells were blocked with 17% donkey serum, 0.3% Triton-X100 in phosphate-buffered saline and then incubated sequentially with 14G2 (mouse anti-AKAP350), mouse anti-p58 (1:85), anti-rabbit IgG (1:2000), anti-goat IgG (1:10000) in 1% nonfat milk/TBS-Tween. Blots were exposed to x-ray film at −70°C for 24 h. Immunocytochemistry—HCA-7 cells were grown on glass coverslips to confluence. The cells were then incubated in the absence of the presence of 10 μg/ml brefeldin A for 1 h at 37 °C. Cells were washed in phosphate-buffered saline and fixed with 4% paraformaldehyde for 15 min at room temperature. Cells were blocked with 17% donkey serum, 0.3% Triton-X100 in phosphate-buffered saline and then incubated sequentially with 14G2 (mouse anti-AKAP350), mouse anti-p58 (1:85), anti-rabbit IgG (1:2000), anti-goat IgG (1:10000) in 1% nonfat milk/TBS-Tween. Blots were finally washed three times with TBS-Tween, blots were incubated with horseradish peroxidase-conjugated anti-mouse IgG (1:2500), anti-rabbit IgG (1:2000), or anti-goat IgG (1:10000) in 1% nonfat milk/TBS-Tween. Blots were finally washed three times with TBS-Tween and three times with TBS, and immunoreactivity was detected with chemiluminescence (SuperSignal, Pierce) and subsequent exposure to BioMax film (Kodak).

**Northern Blot Analysis**—A human multi-tissue Northern blot was purchased from Origenes. The blot was sequentially probed with a 336 nucleotide probe corresponding to the 5′ end of human CLIC5A (nucleotides 1–336; GenBank™ accession number AF216941) or a 516 nucleotide probe corresponding to the 5′ end human CLIC5B (nucleotides 1–516 GenBank™ accession number AF032602). Blots were probed overnight at 42 °C and then washed to high stringency (0.1X SSC, 65 °C). Blots were exposed to x-ray film at −70°C for 24 h. Immunocytochemistry—HCA-7 cells were grown on glass coverslips to confluence. The cells were then incubated in the absence or presence of 10 μg/ml brefeldin A for 1 h at 37 °C. Cells were washed in phosphate-buffered saline and fixed with 4% paraformaldehyde for 15 min at room temperature. Cells were blocked with 17% donkey serum, 0.3% Triton-X100 in phosphate-buffered saline and then incubated sequentially with 14G2 (mouse anti-AKAP350), mouse anti-p58 (1:85), anti-rabbit IgG (1:2000), anti-goat IgG (1:10000) in 1% nonfat milk/TBS-Tween. Blots were washed overnight at 42 °C and then washed to high stringency (0.1X SSC, 65 °C). Blots were exposed to x-ray film at −70°C for 24 h. Immunocytochemistry—HCA-7 cells were grown on glass coverslips to confluence. The cells were then incubated in the absence or presence of 10 μg/ml brefeldin A for 1 h at 37 °C. Cells were washed in phosphate-buffered saline and fixed with 4% paraformaldehyde for 15 min at room temperature. Cells were blocked with 17% donkey serum, 0.3% Triton-X100 in phosphate-buffered saline and then incubated sequentially with 14G2 (mouse anti-AKAP350), mouse anti-p58 (1:85), anti-rabbit IgG (1:2000), anti-goat IgG (1:10000) in 1% nonfat milk/TBS-Tween. Blots were finally washed three times with TBS-Tween and three times with TBS, and immunoreactivity was detected with chemiluminescence (SuperSignal, Pierce) and subsequent exposure to BioMax film (Kodak).

**RESULTS**

Identification of an Interaction of the CLIC Family of Proteins with AKAP350—AKAP350 interacts with several signaling proteins including protein phosphatase 1, protein kinase N, protein phosphatase 2A (8), protein kinase Ce (11) and Type II protein kinase A (Fig. 1A) (7, 8). To investigate further the function of AKAP350 as a scaffolding protein, we performed a yeast two-hybrid screen of a rabbit parietal cell library. We screened the library with a 3.2-kb segment of AKAP350, previously termed the PHR (7) because of its weak homology (41% similarity) with the centrosomal protein, pericentrin (Fig. 1A). One positive clone was identified as a full-length cDNA of the rabbit homologue of human CLIC1 (NCC27) (GenBank™ accession number U93205). Subsequent yeast two-hybrid binary
and PKC of the PHR used to map the CLIC binding region (shown in gray) is indicated on the Rabbit CLIC1 binding to the PHR constructs in yeast two-hybrid assays.

Four leucine zipper regions (LZ) and the binding sites for RII, PKN, PP1, and PKCε. Also shown is the pericentrin homology region. B, constructs of the PHR used to map the CLIC binding region (shown in gray). Rabbit CLIC1 binding to the PHR constructs in yeast two-hybrid assays is indicated on the right.

assays confirmed the interaction of rabbit CLIC1 with the PHR of AKAP350. The rabbit CLIC1 sequence and deletion constructs of the PHR of AKAP350 were used in yeast two-hybrid binary assays to map the rabbit CLIC1 binding site in AKAP350. The CLIC binding region of AKAP350 was limited to a 133-amino acid region between amino acids 1388 and 1515 of AKAP350. The rabbit CLIC1 sequence and deletion constructs of the PHR of AKAP350 were used in yeast two-hybrid binary assays to map the rabbit CLIC1 binding site in AKAP350. The CLIC binding region of AKAP350 was limited to a 133-amino acid region between amino acids 1388 and 1515 of AKAP350 (Fig. 1B).

Coimmunoprecipitation of AKAP350 and CLIC Family Members—To characterize the CLIC/AKAP350 interaction, coimmunoprecipitation experiments were performed using a rabbit polyclonal anti-CLIC antibody, 656. This antibody was chosen for its ability to recognize a range of CLIC family members. The antibody was developed against a region of the carboxyl terminus of the avian CLIC, p62, and is known to recognize avian p62, bovine p64 (17), and human CLIC1 and CLIC4.2

Fig. 2A demonstrates that, in extracts of HCA-7 cells, the pan-CLIC 656 antibody recognized major bands of 31, 32, and 46 kDa as well as a minor band at 85 kDa. Beads with only anti-CLIC antibody or beads coated with the anti-pan-CLIC antibody, 656, were incubated with 1% Nonidet P-40 extracts from HCA-7 human colon adenocarcinoma cells. The bound protein was separated by SDS-PAGE and subjected to Western blot analysis with the monoclonal anti-AKAP350 antibody, 14G2. The anti-CLIC antibody 656 specifically immunoprecipitated AKAP350 when compared with beads coated only with anti-CLIC antibody (Fig. 2B). The Western blot analysis with antibody 656 confirmed that it did not recognize AKAP350 (data not shown). These biochemical data confirmed the in situ association of AKAP350 with a CLIC family member in HCA-7 cells.

CLIC5B: A Novel CLIC Family Member in HCA-7 Cells—As noted in Fig. 2A, the 656 pan-CLIC antibody recognized at least four discrete proteins in HCA-7 cells. The 31- and 32-kDa bands could represent CLIC1 and CLIC4 because of their apparent molecular mass and the known ability of the 656 antibody to cross-react with CLIC1 and CLIC4.2 Previously, no CLIC protein had been described as migrating at 46 kDa. Another pan-CLIC rabbit polyclonal antibody (121), which recognizes the conserved CLIC carboxyl terminus of CLIC1 (14), also recognized a 46-kDa band in HCA-7 cells (data not shown).

We employed several specific CLIC antibodies to determine whether they recognized this protein. Antibodies specific for CLIC1 and parchorin did not recognize this protein (data not shown). However, only a polyclonal antibody affinity-purified against CLIC5 (B132) did recognize a 46-kDa band (data not shown). These results suggested that a CLIC5 immunoreactive protein might represent the 46-kDa CLIC species in HCA-7 cells.

The high homology between bovine p64 and the more recently documented CLIC5 led us to hypothesize that a CLIC5 splice variant might represent a human homologue of bovine p64 (13, 14). Human CLIC5 and bovine p64 show extensive identity in their carboxyl termini but have little similarity in their amino-terminal sequences. We performed a search with the 5′ coding sequence from bovine p64 against genomic BAC sequences containing the human CLIC5 cDNA. This search revealed an alternate initiation site for CLIC5 that is present 65 kb upstream of the published start site of human CLIC5 (Fig. 3). Utilizing a sense primer against this putative alternative start site and an antisense primer against the 3′ end of the CLIC5 coding sequence, we were able to amplify a 1200-bp sequence from HCA-7 cell cDNA (Fig. 3). This sequence encodes for a protein with an apparent molecular mass of 46 kDa, corresponding to the molecular weight of the CLIC-immunoreactive band detected by the 656 antibody (Fig. 2A). We named this protein CLIC5B (GenBankTM accession numberAY032602) and have renamed the previously published CLIC5 as CLIC5A (14). The only difference between these proteins is found in their first exons. The first exon of CLIC5A encodes for its first 21 amino acids, whereas the first exon of CLIC5B encodes for its first 180 amino acids (Fig. 3B). Because human CLIC5B shows 72% identity with bovine p64, CLIC5B most likely represents the human homologue of bovine p64.

Northern Analysis of CLIC5B—To determine the tissue distribution of these two proteins, Northern blot analysis of 12 different human tissues was performed. Due to the CLIC5 splicing, we designed a CLIC5A-specific probe that included a region of the 5′ untranslated sequence and the unique first exon of CLIC5A. The message size of CLIC5A was consistent with the previously published size of 6.4 kb (14) (Fig. 4A). We found the highest expression in the lung, with a lower level of
message in the heart, kidney, and skeletal muscle than previously reported with a probe designed against full-length CLIC5A (13). CLIC5A mRNA was also detected in the colon and placenta upon longer exposure of the blot to film (data not shown). As Berryman and Bretscher (14) described, the CLIC5A-specific probe also recognized less intense bands at 3.8 and 2.3 kb in some tissues (data not shown). A probe designed against the unique first exon of CLIC5B was used to evaluate the tissue distribution of CLIC5B. CLIC5B expression was detected only in the small intestine. The CLIC5B mRNA migrated at a slightly higher size (6.6 kb) than CLIC5A (Fig. 4B).

**CLIC5B Interacts with AKAP350**—The CLIC family of proteins is defined by their high homology in their carboxyl termini, which contain two conserved putative transmembrane domains (18). To determine the specificity of AKAP350 binding for members of the CLIC family, clones of human CLIC1, CLIC4, CLIC5A, CLIC5B, and rabbit parchorin (14, 16) were paired with AKAP350 in yeast two-hybrid binary assays. Each of these CLIC family members interacted with AKAP350 (Fig. 5A). These results suggested that the conserved carboxyl terminus in the CLIC family contained the binding site for AKAP350 (Fig. 5A).

Constructs containing the first and second halves of the conserved region of CLIC5B were used in yeast two-hybrid binary assays to determine the region responsible for binding to AKAP350. The binding region was contained in the final 120 amino acids of CLIC5B. The entire region was necessary for the binding of AKAP350 to CLIC5B, because any further truncations of this region were unable to bind to AKAP350 (Fig. 5B). This region contains extensive identity in all CLIC family members (Fig. 5B).

**CLIC5B Staining Colocalizes with AKAP350 in the Golgi Apparatus in HCA-7 Cells**—In an accompanying paper (19), we have characterized AKAP350A as a Golgi apparatus-specific splice variant of AKAP350. To explore the localization of AKAP350 and CLIC5B, we prepared a goat polyclonal antibody against CLIC5B-specific peptide sequence. Fig. 6 demonstrates that antibodies against CLIC5B detected a single 46-kDa band in fractions of HCA-7 cells with the highest concentrations in heavy microsomes and the high speed superna-

Fig. 3. CLIC5B sequence. A, nucleotide sequence and deduced amino acid sequence of CLIC5B amplified from an HCA-7 cDNA. The highlighted sequence and corresponding amino acids represent the novel first exon of CLIC5B created by an alternate start site upstream of the start site of CLIC5A. B, diagram of the CLIC5 gene in chromosome 6 (GenBank accession no. 13643945) showing the alternate start site of CLIC5B upstream of CLIC5A. Nucleotide numbers corresponding to the following exons are: CLIC5B exon 1, 195221–194681; CLIC5A exon 1, 130521–130458; exon 2, 54373–44262; exon 3, 48510–48380; exon 4, 40780–40674; exon 5, 13537–13355; and exon 6, 2384–2218. The bar represents 10 kb, and the double line breaks represent 20 kb.
AKAP350 and CLIC Family Interaction

**Northern blot analysis of CLIC5A and CLIC5B expression in human tissues.** A, Northern blot analysis of a human multi-tissue blot with a probe created from 5’ untranslated and exon 1 of CLIC5A. B, Northern blot analysis of the same human multi-tissue blot probed with a probe representing the first exon of CLIC5B. Lanes containing mRNA from whole organs are as follows: 1, brain; 2, colon; 3, heart; 4, kidney; 5, liver; 6, lung; 7, skeletal muscle; 8, placenta; 9, small intestine; 10, spleen; 11, stomach; 12, testis.

All CLIC5B immunoreactivity was abolished by incubation with 10 μM competing CLIC5B peptide (data not shown). AKAP350 was also present in these fractions but was also detected in the lighter microsomal fractions.

Because the patterns of AKAP350 and CLIC5B immunoreactivity were overlapping but not identical, CLIC5B antibodies were used to stain HCA-7 cells. Fig. 7A demonstrates that, although considerable cytosolic staining was observed, CLIC5B immunoreactivity localized to the Golgi with extensive colocalization with the p58 Golgi marker. A pan-CLIC antibody (121) also demonstrated strong staining of the Golgi apparatus (data not shown). Treatment of cells with brefeldin A led to disruption of the Golgi and vesiculation of p58 staining with dispersal of CLIC5B staining into a predominantly cytosolic distribution (Fig. 7A). We also observed colocalization of CLIC5B with AKAP350 staining with the 14G2 monoclonal antibody (Fig. 7B). Brefeldin A treatment dispersed the Golgi staining by both CLIC5B and AKAP350 antibodies. However, in brefeldin-treated cells, it was also apparent that a small amount of CLIC5B staining was also present at centrosomes in which AKAP350 staining was unaltered by brefeldin treatment (Fig. 7B). These results demonstrate that CLIC5B localizes predominantly to the Golgi apparatus, with a lesser association with the centrosome.

**GFP-CLIC5B-(178–410) Targets to the Golgi Apparatus—**To clarify the localization of CLIC5B with the Golgi apparatus, a GFP-chimera (GFP-CLIC5B-(178–410)) was constructed utilizing amino acids 178–410 of CLIC5B. This region represents the shared exons 2–6 of CLIC5A and CLIC5B (Fig. 4). The HCA-7 cells were then stained with the Golgi apparatus marker p58 (Fig. 8). HCA-7 cells expressing GFP-CLIC5B-(178–410) displayed colocalization with the Golgi apparatus. Staining with the anti-AKAP350 14G2 antibody was unchanged in cells expressing this GFP-chimera (data not shown). Treatment of cells with brefeldin A caused dispersal of GFP-CLIC5B-(178–410), confirming its localization to the Golgi apparatus (data not shown). These data suggest that the region coding for exons 2–6 is sufficient for the targeting of CLIC5B to the Golgi apparatus.

**DISCUSSION**

AKAPs scaffold signaling proteins to specific locations required for specialized functions throughout cells. The signaling proteins associated with an AKAP often define the functions of these scaffolds (5). In this report, we have identified the interaction of AKAP350 with CLIC family members, specifically the colocalization of CLIC5B and AKAP350 at the Golgi apparatus of HCA-7 cells. For the first time, these results demonstrate the interaction at the Golgi apparatus of AKAP350 with an effector protein with no known enzymatic activity. In addition, these studies establish a mechanism for the scaffolding of CLIC proteins at discrete locations within the cell.

The CLIC protein family consists of seven members, all of which have several common attributes. The family consists of parchoerin, bovine p64, and CLIC1-CLIC5. Each protein in this family has a high amino acid homology at the carboxyl-terminal end of the protein. This homologous region consists of ~200 amino acids and contains a putative hydrophobic membrane-spanning domain. Although expression patterns and localization differ for each of the family members, all of these CLIC proteins associate with membranous organelles.

The data presented here suggest that the CLIC5 gene encodes for at least two gene products. Northern blot analysis for CLIC5A and CLIC5B also demonstrated different mRNA expression patterns for these proteins. Multiple message sizes have been reported for CLIC1, CLIC4, CLIC5A, and p64 (13, 14, 20–22), suggesting that each of the members of this family of proteins contains several multiply spliced species. Because of the high homology among CLIC family members in their carboxyl termini, amino-terminal region splicing events could provide distinguishing characteristics enabling a single gene to encode for proteins with a diverse range of functions. Berryman and Bretscher (14) utilized the full-length CLIC5A cDNA as a probe to perform Northern analysis of a multi-tissue blot. To distinguish CLIC5A from CLIC5B, we utilized probes specific for the unique first exons of CLIC5A and CLIC5B. The CLIC5B probe recognized a single 6.6-kb message detectable only in the small intestine. The CLIC5B message was slightly longer than the CLIC5A message of 6.4 kb observed in several tissues (14). Interestingly, the CLIC5A-specific probe still recognized other shorter message sizes of 3.8 and 2.3 kb (data not shown), which correlates with the reported mRNA sizes observed with the probe designed against full-length CLIC5A (14). These smaller mRNAs could represent shorter message sizes created from splicing events downstream of the first exon. The bovine homologue of CLIC5B (p64) also showed similar multiple message sizes by Northern analysis (13). The differences observed between each of these studies warrants further investigation to determine the functional diversity of CLIC5/p64 splice variants.

Previous studies have demonstrated the association of CLIC family members with the cytoskeleton (CLIC5A), nucleus (CLIC3, CLIC1), Golgi apparatus (bovine p64), mitochondria (CLIC4), and the sub-apical membrane compartment (CLIC1, CLIC4, CLIC5A, and parchoerin) (13, 14, 16, 20, 21, 23). Targeting domains in CLICs responsible for their localization have not been elucidated. The 46-kDa CLIC5B protein appears to be a major immunoreactive CLIC in HCA-7 cells. Immunocytochemical studies demonstrated the predominant localization of CLIC5B immunoreactivity with the Golgi apparatus in HCA-7 cells. CLIC5B, formed through an alternate initiation site of the CLIC5A gene, most likely represents the human homologue of bovine p64. CLIC5B and p64 share 72% identity; p64 was originally isolated from kidney microsomes containing Golgi apparatus markers (13). Interestingly, the region conserved in CLIC5A and CLIC5B (exons 2–6) localized to the Golgi apparatus when overexpressed as a GFP-chimera protein in HCA-7 cells. Therefore, the ability to localize to the Golgi apparatus is at least partially maintained in the conserved region of CLIC5A and CLIC5B. Previous studies have localized CLIC5A predominantly to the supapical compartment (14). The first exon of CLIC5A may therefore code for a motif required for Golgi apparatus export or targeting to the apical membrane.
domain. Although AKAP350 can bind to CLIC5B and could sequester it to the Golgi apparatus, we have shown that AKAP350 can potentially bind to all CLIC family members. Therefore, interactions with AKAP350 may not be sufficient for organelle-specific targeting of CLIC family members. The association of AKAP350 and CLIC5B was affected by structural changes in the Golgi apparatus. In brefeldin A-treated HCA-7 cells, both CLIC5B and AKAP350 staining were dispersed in the cytosol. These data suggest that the structural integrity of the Golgi apparatus is necessary for the association of CLIC5B and AKAP350 with the Golgi cisternae. These results support a model for AKAP350 scaffolding of CLIC5B at

Fig. 5. **AKAP350 interaction with CLIC.** A, diagram representing parchorin, CLIC1, CLIC4, CLIC5A, and CLIC5B, which all interact with AKAP350 in yeast two-hybrid assays (indicated on the right). Constructs of the conserved region (gray) in CLIC5B were used to define where AKAP350 binds. The last 120 amino acids (290–410) were able to interact with AKAP350 but deletion constructs of this region were unable to bind. B, carboxyl-terminal AKAP350 binding domain of CLIC family proteins. Protein alignment of the last 120 amino acids of CLIC5B, which bind to AKAP350, are compared with parchorin, CLIC1, CLIC4, CLIC5A, and p64 bovine. The highlighted regions represent homology as compared with CLIC5B. A putative transmembrane domain is underscored with Xs.
the assembled trans-Golgi cisternae. In addition, Western analyses demonstrate a large amount of both CLIC5B and AKAP350 protein in the supernatant fraction after homogenization. These data suggest a dynamic association of CLIC5B with AKAP350, providing a mechanism for the modulation of CLIC function at the Golgi apparatus.

Although the function of CLIC5B at the Golgi apparatus is unclear, previous studies have described CLIC family members as independent channels. Harrop et al. (24) have recently described a 23-amino acid sequence in carboxyl terminus of CLIC1 as a putative transmembrane domain. Indeed, this region is highly conserved among all of the CLIC family members. A mounting volume of evidence describes the CLIC proteins as potential integral membrane proteins that can exist as cytosolic and/or membrane proteins based on a significant conformational change in the amino terminus (24, 25). This new conformation would expose the putative transmembrane domain. The large conformational change required in CLIC1 for the exposure of this domain could require signaling through kinases and phosphatases. Because AKAP350 contains putative binding sites for protein phosphatase 1, protein kinase N, protein phosphatase 2A (8), protein kinase C/H9280 (11), phosphodiesterase 4D3 (12), and protein kinase A (7, 8), it is conceivable that these enzymes could play a role in the function of CLIC5B as well as other CLIC family members. Edwards and colleagues (26) have suggested that phosphorylation and dephosphorylation of the CLIC5B bovine homologue, p64, has an effect on its ability to control chloride movement. Further investigations into the function of CLIC5B as a chloride anion channel will have to be completed before it can be determined whether this CLIC protein mediates chloride movement at the Golgi apparatus.

Signaling events will likely control the movement and function of the CLIC family of proteins. In this report, we describe the interaction of all CLIC family members with a conserved region found in all AKAP350 splice variants (7). Therefore, it is possible that other CLIC family members will be scaffolded by AKAP350 in other intracellular locations in particular cell types. Although we have not established that AKAP350 is present wherever CLIC family members are localized, a more comprehensive study of other cell types could lead to the discovery of other AKAP350/CLIC interactions. This interaction is intriguing because of the diversity of localized signaling molecules that AKAP350 can potentially target to control the function of CLICs. Many of the CLIC family members share phosphorylation motifs for enzymes scaffolded by AKAP350. For example CLIC4, p64, CLIC5A, and CLIC5B all share a conserved protein kinase A phosphorylation site in their extreme carboxyl termini (27, 28). Thus, cAMP-dependent phosphorylation of CLIC proteins by protein kinase A could regulate their distribution and function.

In summary, we have described the interaction of CLIC...
family members with AKAP350. In HCA-7 cells, CLIC5B, the human homologue of bovine p64, colocalizes predominantly with AKAP350 at the Golgi apparatus with lesser localization at the centrosome. This report represents the first demonstration of AKAP350 with a non-enzymatic effector protein at the Golgi apparatus. These studies indicate that AKAP350 not only scaffolds enzymatic signaling molecules but also conveys organelle-specific interactions with non-kinase/phosphatase effector molecules. The relationships between the signaling proteins scaffolded to AKAP350 and CLIC proteins will provide further insights into the function of the CLIC family of proteins and AKAP350 in specific subcellular compartments.

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