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ORIGINAL ARTICLES

Localization of the Nucleic Acid Channel Regulatory Subunit, Cytosolic Malate Dehydrogenase

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Abstract NACh is a nucleic acid–conducting channel found in apical membrane of rat kidney proximal tubules. It is a heteromultimeric complex consisting of at least two proteins: a 45-kDa pore-forming subunit and a 36-kDa regulatory subunit. The regulatory subunit confers ion selectivity and influences gating kinetics. The regulatory subunit has been identified as cytosolic malate dehydrogenase (cMDH). cMDH is described in the literature as a soluble protein that is not associated with plasma membrane. Yet a role for cMDH as the regulatory subunit of NACh requires that it be present at the plasma membrane. To resolve this conflict, studies were initiated to determine whether cMDH could be found at the plasma membrane. Before performing localization studies, a suitable model system that expressed NACh was identified. A channel was identified in LLC-PK_1 cells, a line derived from pig proximal tubule, that is selective for nucleic acid and has a conductance of approximately 10 pS. It exhibits dose-dependent blockade by heparan sulfate or L-malate. These characteristics are similar to what has been reported for NACh from rat kidney and indicate that NACh is present in LLC-PK_1 cells. LLC-PK_1 cells were therefore used as a model system for immunolocalization of cMDH. Both immunofluorescence and immunoelectron microscopy demonstrated cMDH at the plasma membrane of LLC-PK_1 cells. This finding supports prior functional data that describe a role for cMDH as the regulatory subunit of NACh.

Keywords Artifical planar membranes · Electrophysiology · Epithelial iontransport · Ion channels · Ion transport by epithelia · Membranebiology/immuno chemistry · Membrane transport · Planarbilayers · Renal physiology · Ion channels

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After observations that purified poliovirus RNA could "infect" cultured cells and lead to viral protein production, investigators have attempted to define the mechanism of nucleic acid entry into the cell. After nearly 40 years of research, the mechanisms remains poorly defined, but two primary processes have been proposed: endocytosis (either fluid phase or receptor mediated) and a saturable nonendocytic pathway. A number of cell surface binding proteins have been identified that are postulated to mediate the endocytic pathway, and we have recently identified a complex of proteins that mediates the endocytosis-independent pathway.

In 1998, we purified a highly specific nucleic acid conducting channel from renal brush border membranes (Hanss et al. 1998). This channel conducts single-stranded nucleic acids with a conductance of 10 pS and does not show rectification or voltage dependence. The channel is not voltage dependent and does not require metabolic energy. We have shown that the nucleic acid–conducting channel (NACh) is a heteromultimeric complex with two functional subunits: a pore-forming subunit and a regulatory subunit. Recent studies have shown that the pore-

forming subunit, when reconstituted alone in planar lipid bilayers, forms a nonselective pore that does not require nucleic acids to gate and conducts a number of ions, including potassium, chloride, and oligodeoxynucleic acids (ODN). The regulatory subunit does not form a conductive pathway when reconstituted alone; however, when reconstituted with the pore-forming subunit, the channel is converted to a nucleic acid–dependent, nucleic acid–conducting channel. Recent studies in our laboratory have identified the regulatory subunit as cytosolic malate dehydrogenase (cMDH) (Hanss et al. 2002).

Involvement of cMDH in regulation of the nucleic acid channel requires that at least a subpopulation must be present in or near the plasma membrane. To our knowledge, this localization has not been described in the literature. For this reason, we initiated experiments to determine whether cMDH is present at the plasma membrane.

Methods

Cell Line and Anti-cMDH Antiserum

Immunolocalization studies were carried out in LLC-PK_1 cells, a renal cell line derived from porcine kidney. This is a well-established cell line that has been used extensively to study renal transport processes. Cells were maintained in T-75 plastic flasks and fed medium M199 (Gibco/BRL-Invitrogen) supplement with 3% fetal bovine serum, glutamine (2 mM), penicillin (100 units/ml), and streptomycin (100 μg/ml). For channel purification studies, cells were grown to confluence, detached from the flask by scraping, pelleted, and stored at −70°C until use. Purification experiments were begun when we had collected approximately 10 g of cells.

For immunolocalization studies, LLC-PK_1 cells were seeded onto collagen (type I collagen from rat tail; BD Biosciences, Bedford MA)-coated glass coverslips (∼10,000 cells) and grown until 90% confluent. We have previously reported generation of a polyclonal mouse serum that is immunoreactive with cMDH (Hanss et al. 2002). Mice were immunized with pig heart cMDH (Sigma Chemical Co., St. Louis, MO), and serum was collected by standard protocols.

Identification of the Nucleic Acid Channel in LLC-PK_1 cells

Before cMDH localization studies were performed in LLC-PK_1 cells, the presence of NACh in these cells was verified. To accomplish this, methods were adapted from previous studies (Hanss et al. 1998, 2002). Briefly, LLC-PK_1 cells were grown to confluence and apical membranes were isolated by a multistep differential centrifugation protocol. The apical vesicles were then subjected to affinity purification with an antibody raised against p45, as previously described (Hanss et al. 2002). Fractions containing channel activity were analyzed by sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE).

Channel activity was assessed in each fraction and with the final purified protein sample by reconstituting protein samples in planar lipid bilayer. Proteins were reconstituted by sonicating purified protein (80 kHz for 1 min) with a 1:1 mixture of phosphatidylethanolamine (10 mg/ml) and phosphatidylserine (10 mg/ml; Avanti Polar Lipids) to form proteoliposomes. The proteoliposomes were then allowed to fuse with preformed planar lipid bilayers consisting of the same lipids use to form proteoliposomes. The lipid bilayers were formed between two solution chambers (1 ml each), which were filled with a buffered solution consisting of 200 mM CsCl, 20 mM HEPES, and 1 mM $CaCl_2$, pH 7.4. A 20-mer homomultimer of deoxythimidine (poly-dT_{20}) was added to the solution chambers to a concentration of 5 μM. The solution chambers were attached to a patch clamp amplifier (Axopatch 200B, Axon Instruments) with the cis chamber connected to the amplifier and all voltages referenced to the trans (ground) side. Data were digitized, filtered, and analyzed by commercially available software (PCLAMP, Version 9.1, Axon Instruments).

Immunofluorescence Studies

LLC-PK_1 cells were plated on collagen (type I collagen from rat tail; BD Biosciences, Bedford, MA)-coated glass coverslips, and when the cells were approximately 90% confluent, cMDH was localized by immunofluorescence by means of methodology adapted from Rappoport et al. (2001). Briefly, cells were fixed for 2 min in ice-cold 2% paraformaldehyde, 4% sucrose in phosphate-buffered saline (PBS), washed in PBS, permeabilized with Triton X-100 (0.1%), and then washed again in PBS. Cells were incubated at room temperature for a minimum of 30 min in a blocking solution consisting of 2% fetal bovine serum, 2% bovine serum albumin (BSA), and 0.2% fish gelatin and then in blocking solution containing polyclonal antiserum raised against cMDH (diluted 1:200). After a minimum of 1 h, cells were washed in ice-cold PBS, incubated in Cy2-conjugated secondary antibody (1:200) for 60 min, washed, and mounted on glass slides. In some experiments, cell membranes were visualized with Texas Red–conjugated wheat germ agglutinin (WGA). In these experiments, cells were first stained with cMDH antiserum and then incubated with WGA (as per manufacturer's instructions) for 30 min at 37°C. Cells were then rinsed once in PBS and fixed in 4% paraformaldehyde for 5 min

at room temperature. After immunolabeling, cells were examined with a Leica TCS-SP1(UV) confocal laser scanning microscope (Leica Microsystems, Heidelberg, Germany). The AOTF was adjusted for each laser line such that there was no cross talk between detected channels. Illumination was provided by the 488-nm line of an Ar laser and a 568-nm Kr laser. Images were collected with a 100×, 1.4 n.a. Plan-Apo oil immersion lens.

Cell Fractionation

To determine whether cMDH can be found at nuclear membrane, we fractionated LLC-PK_1 cells and performed Western blot analysis on each fraction with a cMDH antibody. Fractionation was performed with the Proteo-Extract subcellular extraction kit (Calbiochem) according to the manufacturer's protocol.

Enhanced Green Fluorescent Protein (EGFP) Fusion Protein

To provide additional evidence for cMDH in plasma membrane and to examine possible nuclear localization of cMDH, a plasmid was constructed that placed EGFP in frame with and downstream of cMDH. LLC-PK_1 cells were grown on collagen-covered coverslips and transfected with this plasmid with Lipofectamine 2000 (Invitrogen). Approximately 18 h later, cells were fixed, coverslips were mounted on glass slides, and EGFP fluorescence was detected by confocal microscopy.

Immunoelectron Microscopy

To confirm localization determined by immunofluorescence studies, experiments were performed to immunolocalize cMDH by electron microscopy. LLC-PK_1 cells were grown on Thermanox coverslips (Electron Microscopy Sciences, Fort Washington, PA) to approximately 80% confluence and fixed in 3.0% paraformaldehyde plus 0.5% glutaraldehyde in 10 mM phosphate buffer pH 7.4 for 60 min. After fixation, cells were washed with 10 mM phosphate buffer, pH 7.4 followed by washes with double-distilled water. Cells were then dehydrated through a graded series of ethanol (15 min each in 50%, 70%, and 95% ethanol). After dehydration, cells were infiltrated with two parts 90% ethanol and one part Lowicryl K4 M (Electron Microscopy Sciences, Fort Washington, PA) for 30 min, followed by two parts Lowicryl and one part ethanol for 30 min, and finally two changes (30 min each) of 100% Lowicryl. The Thermanox coverslips were cut in half and flat embedded in Lowicryl, polymerized at −21°C under ultraviolet light for 24 h, and then further polymerized for 3 days at room temperature.

For immunoelectron microscopy, sections were cut and collected on formvar-coated slot nickel grids, floated on a drop of 0.05 M glycine in PBS for 15 min at room temperature, drained, and transferred to blocking solution (5% BSA, 0.1% cold-water fish gelatin, and 5% normal goat serum in PBS, pH 7.4) for 30 min. The sections were washed in PBS and incubated overnight on a drop of anti-cMDH polyclonal antibody (1:50) in PBS, pH 7.4 with 0.2% BSA. Sections were washed in PBS with 0.2% BSA and floated on colloidal gold (10 or 20 nm) conjugated goat anti-mouse IgG (1:100) in PBS with 0.2% BSA for 30 min. Sections were then washed as before, postfixed with 2% glutaraldehyde in PBS, washed for 5 min in PBS pH 7.4, and washed twice in double-distilled water. Sections were then stained with uranyl acetate (half saturated in 100% ethanol), for 5 min and Reynold's lead citrate for 3 min and viewed and photographed on a Hitachi H 7000 transmission electron microscopy operated at 75 kV.

Results

Identification of the Nucleic Acid Conducting Channel in LLC-PK_1 Cells

Membrane vesicles purified from LLC-PK_1 cells were subjected to affinity purification protocol. Fractions from the column were collected, assayed for NACh activity, and analyzed by SDS-PAGE. As can be seen in Fig. 1a, an active fraction contained a 45-kDa protein consistent with the pore-forming subunit (lane 2).

The electrophysiologic characteristics of the channel purified from LLC-PK_1 cells are seen in Fig. 1b–d. When reconstituted, these proteins formed a channel that gates only in the presence of oligodeoxynucleic acids (Fig. 1b). These experiments were performed by using a 20-base homomultimer of thymidine (dT_{20}). The channel conducts current linearly between 30 and −30 mV, without signs of rectification (Fig. 1c) or voltage dependence (Fig. 1d).

Pharmacological Characterization of LLC-PK_1 Cell Channel

We have shown previously that NACh purified form rat kidney cortex is blocked by heparan sulfate and L-malate (Hanss et al. 1998, 2002). We therefore tested the sensitivity of the LLC-PK_1 channel to these agents (Fig. 2). We first tested the effects of heparan sulfate, which is known to block rat kidney NACh from the trans side (extracellular side) of the lipid bilayer. Consistent with previous reports, 50 μM heparan sulfate resulted in complete blockade of the LLC-PK_1 channel activity from the trans side but not when applied to the cis side (Fig. 2a). Similarly, L-malate, the

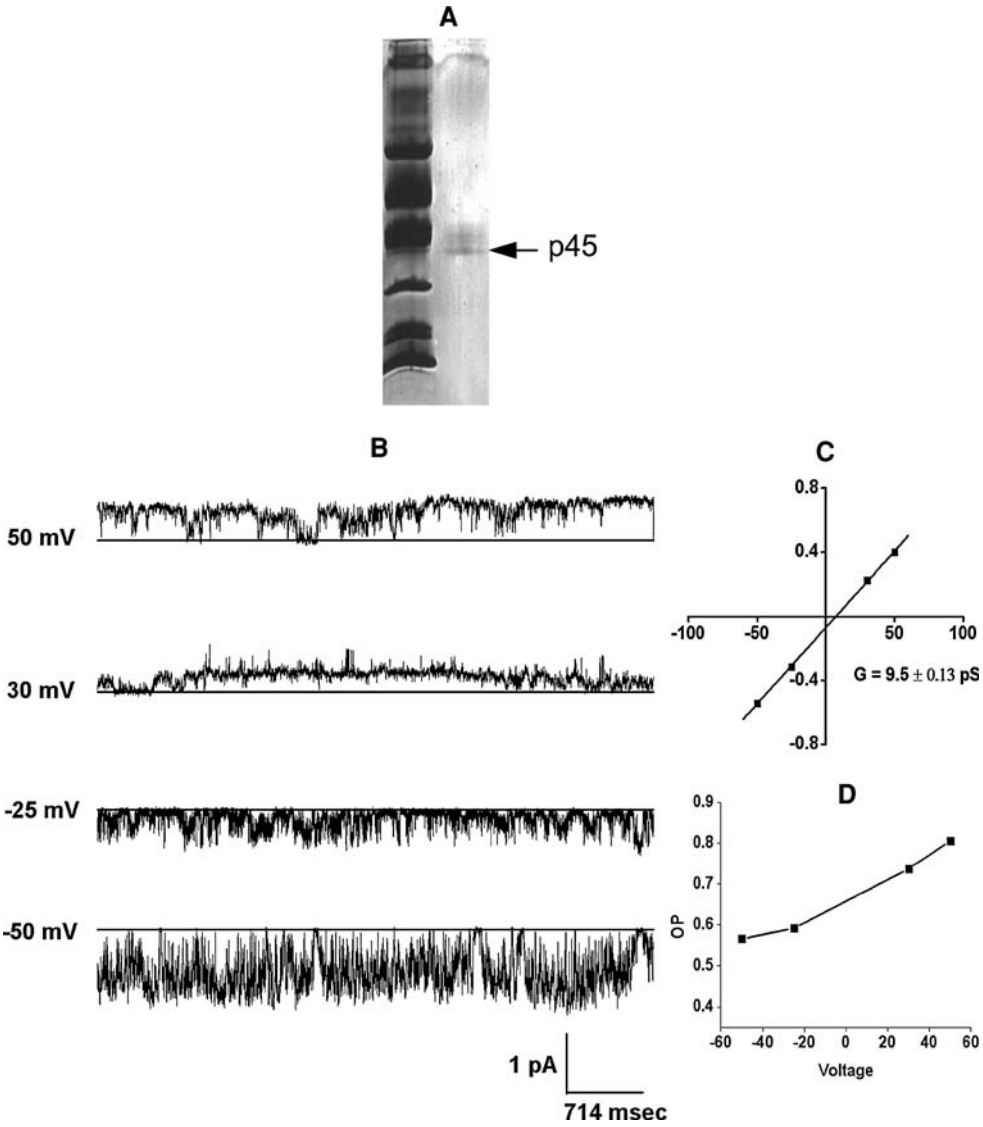

Fig. 1 Identification of NACh in plasma membrane of LLC-PK_1 cells. The presence of NACh was examined in LLC-PK_1 cells, a pig kidney–derived cell line. These cells were selected for two reasons: we have purified NACh from pig kidney proximal tubule and they are a well-established model for the study of transepithelial transport. **a** SDS-PAGE of affinity-purified protein (lane 2) Molecular mass standards are shown in lane 1. Affinity chromatography resulted in purification of a protein band with apparent molecular mass of 45 kDa. This is the expected size for the pore-forming subunit of NACh. **b** Functional reconstituted of a fraction containing p45 and p36 in an artificial planar lipid bilayer revealed a nucleic acid dependent channel activity. **c** Current–voltage relationship for this channel showing a slope conductance of 25 pS. **d** Graph of open probability versus holding potential showing that the channel is not voltage dependent

substrate for cMDH, has been shown to block rat kidney NACh from the cis side (cytoplasmic side) of the lipid bilayer. Increasing amounts of L-malate added to the cis side resulted in concentration-dependent reduction in channel activity (Fig. 2b), whereas addition of L-malate to the trans side was without effect (data not shown).

Together, the data depicted in Figs. 1 and 2 provide both electrophysiological and pharmacological evidence that the nucleic acid channel is present in the plasma membrane of LLC-PK_1 cells.

Localization of cMDH in LLC-PK_1 Cells by Immunofluorescence

If cMDH participates in NACh function, as our previous studies indicate (Hanss et al. 2002), we hypothesized that it should be present at the plasma membrane. We used a murine polyclonal antibody to colocalize cMDH with cellular membranes stained with WGA and analyzed by confocal microscopy as described in Methods. WGA is a carbohydrate-binding protein that binds sialic acid and N-acetylglucosaminyl sugar moieties, which are found in plasma membrane, nuclear envelope, and membranes of the endoplasmic reticulum and Golgi apparatus. WGA labeling in LLC-PK_1 is consistent with this distribution (Fig. 3, top row). In three experiments, cMDH immunostaining was observed in the nucleus, and in patterns consistent with Golgi apparatus, endoplasmic reticulum, and plasma membrane (Fig. 3, middle). When these images are merged (Fig. 3, bottom row), colocalization (in yellow) between lipid membranes, as indicated by WGA, and cMDH are clearly evident at the plasma membrane, in an intracellular reticulated pattern consistent with endoplasmic reticulum, and in a polarized perinuclear pattern

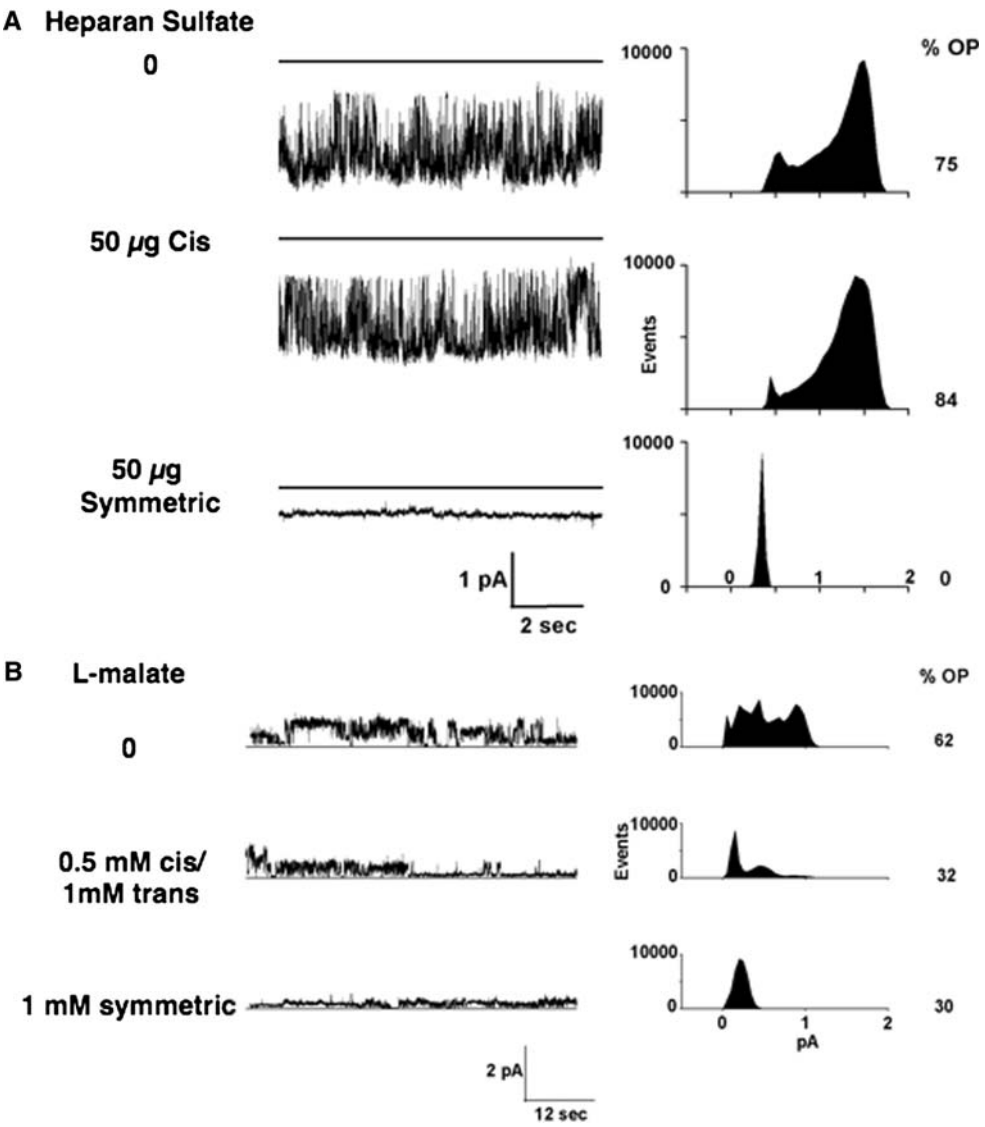

Fig. 2 Pharmacological characteristics of the LLC-PK_1 channel. To determine whether the LLC-PK_1 channel shares pharmacological properties with NACh purified from rat kidney, alterations in channel activity were examined in the presence of heparin sulfate (**a**) or L-malate (**b**), both of which are known to block NACh. Heparan sulfate at a concentration of 50 μg/ml applied to the trans side (extracellular side) of the bilayer blocked nearly all of the nucleic acid dependent channel activity (**a**). Similarly, 1 mM of L-malate significantly reduced both channel open probability and conductance when it was added to the solution bathing the cis side (intracellular side) of the bilayer. These data are consistent with published data for rat kidney–derived NACh

consistent with Golgi staining. It is unclear from these data whether cMDH colocalizes with WGA to the nuclear envelope. This distribution of cMDH within the cell supports our hypothesis that cMDH is the regulatory subunit of NACh by placing it at the plasma membrane.

Investigation of cMDH Localization in Nucleus

Because immunofluorescence data in Fig. 3 show an intense nuclear staining, and because nuclear staining is a fairly common artifact of immunofluorescence staining, additional experiments were performed to confirm or rule out nuclear cMDH. Two approaches were used for these studies: Western blot analysis of subcellular fractions and localization with a cMDH-EGFP fusion protein. Cells were fractionated as described above into cytoplasmic, plasma membrane, nuclear, and cytoskeletal fractions (Fig. 4a, lanes 1, 2, 3, and 4, respectively). A prominent cMDH band is present in the cytoplasmic fraction, and a faint band can be seen in the plasma membrane fraction. cMDH immunoreactivity was not detected in nuclear or cytoskeletal fractions. In an additional series of experiments, LLC-PK_1 cells were transfected with a plasmid expressing a cMDH-EGFP fusion protein. Approximately 18 h after transfection, cells were fixed and viewed by confocal microscopy. As seen in Fig. 4b, EGFP fluorescence was seen in a pattern consistent with that observed in immunofluorescence studies, with the notable exception that there was no EGFP fluorescence associated with nuclei. Together, these data indicate that cMDH is not found in the nucleus of LLC-PK_1 cells and suggest that the nuclear staining seen in immunofluorescence studies was an artifact.

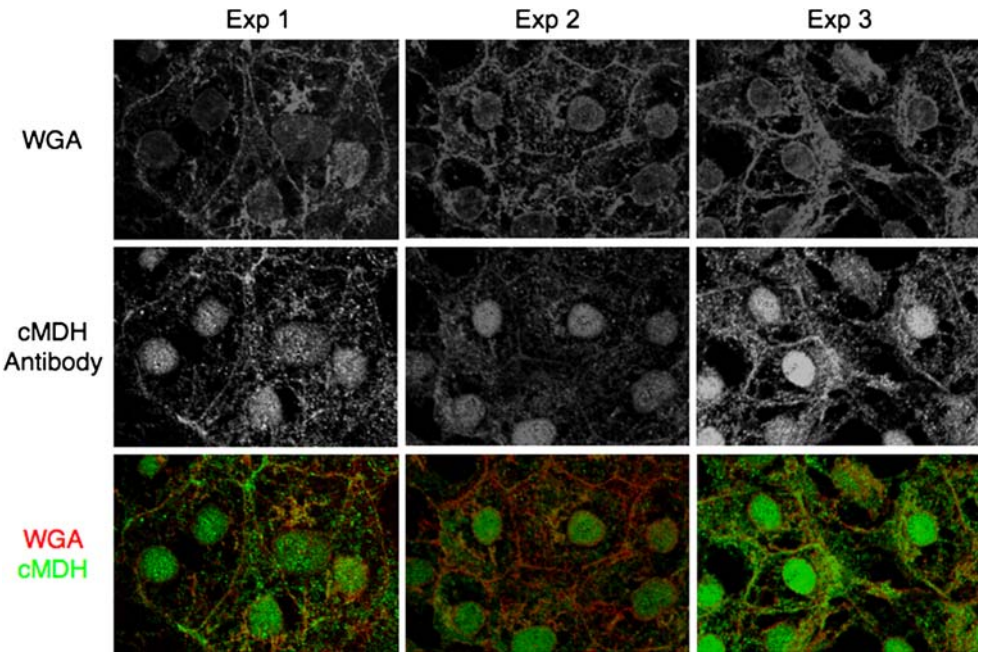

Fig. 3 Immunofluorescence localization of cMDH. Three experiments summarizing cMDH localization using a cMDH specific antiserum (**middle**) and comparing this with the membrane specific dye WGA (**top**). Merged images are shown in the bottom row with WGA staining in red and cMDH staining in green. These data show cMDH immunofluorescence associated with a number of intracellular membranes including what seems to be plasma membrane. Scale bar = 15 μm

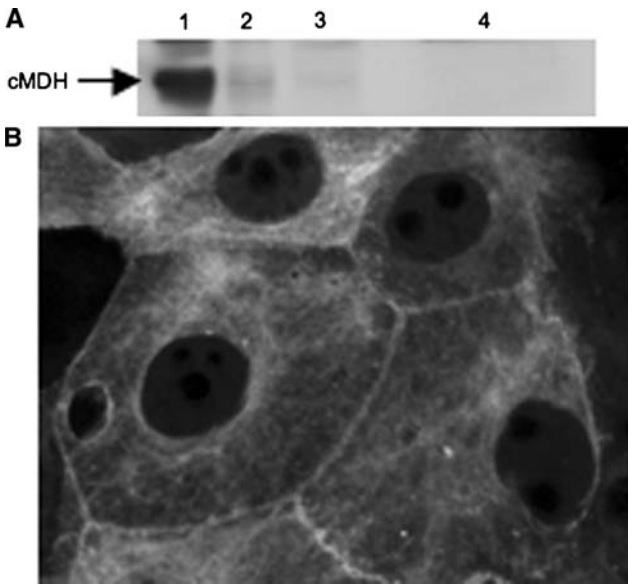

Fig. 4 Cytosolic MDH does not localize to nucleus. **a** Western blot analysis of LLC-PK$_1$ subcellular fractions. Collected fractions are: cytoplasm (lane 1), plasma membrane (lane 2), nucleus (lane 3), and cytoskeleton (lane 4). Immunoreactivity with a cMDH polyclonal antibody was seen in cytoplasm and plasma membrane fractions but not in nucleus or cytoskeleton. **b** cMDH was localized in LLC-PK$_1$ cells using a cMDH-EGFP fusion protein. Intranuclear fluorescence is not seen in these cells. It is important to note that cMDH plasma membrane staining is clearly discernible in this figure

Localization of cMDH in LLC-PK$_1$ Cells by Immunoelectron Microscopy

To confirm cMDH localization in LLC-PK$_1$ cells, studies were performed with immunoelectron microscopy. In Fig. 5 two different LLC-PK$_1$ cells are shown that were stained with cMDH antiserum. Immunogold labeling can be seen in microvilli of the cells (arrows) and in regions of the plasma membrane between the microvilli (arrowheads).

Discussion

These studies were initiated to establish a cell culture model for the study of nucleic acid channel function and to localize cMDH within the cell. Localization of cMDH in the cell is critically important to our overall hypothesis about nucleic acid transport across the plasma membrane because our current model of transport requires cMDH is present at the plasma membrane where it regulates NACh selectivity. The preponderance of literature on cMDH, however, suggests that it is cytoplasmic and not associated with any cellular membranes. Furthermore, available crystal structure of cMDH indicates that it is a globular molecule with its hydrophobic domains packed in interior folds of the protein, where they are well protected from the aqueous environment. This structure also supports a cytoplasmic localization for cMDH. Our functional data, however, suggest a plasma membrane localization.

We have clearly shown that a nucleic acid conducting channel can be purified from brush border membranes of rat kidney proximal tubules (Hanss et al. 1998; Rappaport et al. 1995). We have also shown that this channel can be separated into two functional subunits: a pore-forming subunit and a regulatory subunit. The regulatory subunit is responsible for channel selectivity, and it modifies channel gating kinetics (Hanss et al. 2002). Protein sequencing and

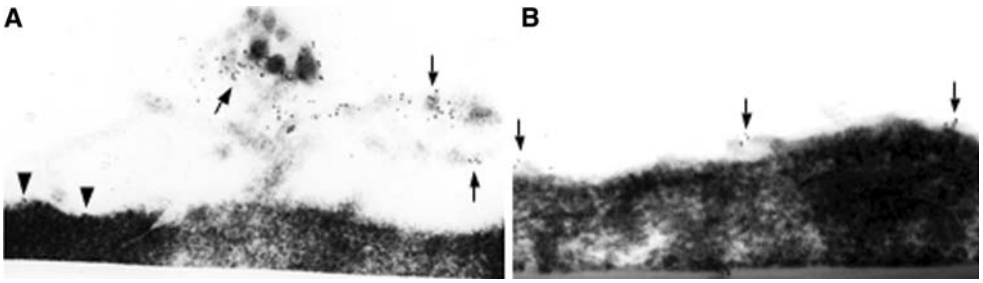

Fig. 5 Immunogold labeling of LLC-PK$_1$ cells with cMDH specific antiserum. LLC-PK$_1$ cells were grown and processed from immunoelectron microscopy as described in Methods. Two different cells are depicted: a dense accumulation of microvilli (**a**) and three individual microvilli (**b**). In both cases, immunogold particles can be seen associated with microvilli (*arrows*) as well as in intervillar regions of the plasma membrane. These data localize cMDH to plasma membrane of LLC-PK$_1$ cells

functional studies definitively demonstrated that cMDH is the regulatory subunit of the channel (Hanss et al. 2002). These functional data, therefore, require cMDH to be located at the plasma membrane where it can interact with the pore-forming subunit.

Before cMDH localization studies could be performed, an appropriate cell culture model of NACh needed to be validated. We choose to examine LLC-PK$_1$ cells as a potential model system. These cells were selected in part because they were originally derived from pig kidney proximal tubules and retain important phenotypic characteristics of proximal tubule. We have shown that NACh is present in acutely isolated pig kidney tissue (unpublished observation). We therefore chose to focus our efforts on these cells.

To identify NACh in any tissue or cell line, criteria were established on the basis of previously observed characteristics of the rat kidney channel (Hanss et al. 1998, 2002; Leal-Pinto et al. 1996). These criteria include: (1) channel activity only in the presence of ODN; (2) channel kinetics comparable to those observed for protein purified from rat kidney; (3) blockade of the channel by heparan sulfate; and (4) blockade of the channel by L-malate. Herein, we present data that show that a nucleic acid–dependent channel can be purified from LLC-PK$_1$ cells. This channel has kinetics parameters that are comparable, but not identical, to that reported for NACh from rat kidney cortex. We also show that this channel is inhibited by heparan sulfate and by L-malate. These data led us to conclude that NACh is present in the plasma membrane of LLC-PK$_1$ cells and allowed us to investigate the localization of NACh in this cell line.

Localization of cMDH by using antibodies against that protein has shown clear membrane association by both fluorescence and electron microscopy. These observations, together with functional data from use of L-malate and cMDH antibodies to block NACh activity in LLC-PK$_1$ cells and in freshly isolated rat kidney brush border membranes, provide a compelling argument supporting our hypothesis. cMDH immunofluorescence is visible in other locations within the cell as well, including intranuclear and cytoplasmic locations. This intracellular distribution of cMDH is consistent with published biochemical localization of cMDH (Aranda et al. 2006; Christie and Judah 1953; Englard and Breiger 1962; Leroux et al. 2006; Penney and Kornecki 1973; Sautter and Hock 1982; Shonk and Boxer 1964). The current data, together with these published studies, have led us to hypothesize that there are two intracellular pools of cMDH, one present in the cytoplasm and a second pool associated with the plasma membrane. Because cMDH does not contain a known membrane localization signal, we further hypothesize that cMDH is targeted to the plasma membrane through its interaction with other NACh proteins, perhaps via p45. Studies are underway to determine the basis of cMDH trafficking to the plasma membrane.

Although the classic view of cMDH localization has not included the plasma membrane, indications of its association with cellular membranes have been suggested in a few publications. For instance, in 1983, Courtneidge et al. identified a 36-kDa substrate (p36) for tyrosine-specific protein kinases that was tentatively identified as cMDH. They also found that more than 60% of p36 resided in a nonsoluble membrane fraction. After this publication appeared, however, there was some controversy about the identification of p36 as cMDH. In another study, a small amount of malate dehydrogenase activity ($\sim$2% of total activity) was found in microsomes, synaptosomes, and synaptic membranes purified from rat brains (Franco et al. 1986). Finally, one study found approximately 10% of MDH activity in an insoluble fraction. These authors, however, did not rule out the presence of mitochondria in the insoluble fraction (Vriezen and van Dijken 1998). Although these studies all support a subpopulation of cMDH in cellular membranes, none provides definitive evidence. The data presented here represent the strongest evidence to date that cMDH is present in the plasma membrane.

One of the more striking features seen in Fig. 3 is the intense fluorescence in the nucleus. Spurious nuclear signals are not uncommon in immunofluorescence studies,

and this could represent such an artifact. Two additional approaches to localize cMDH did not support nuclear localization of cMDH and suggest the nuclear immunofluorescence is an artifact.

The current studies provide evidence that cMDH is associated with plasma membrane. Whether it is an integral membrane protein remains to be determined. We hypothesized in a previous publication that cMDH is an integral membrane protein with two membrane-spanning domains (Hanss et al. 2002). This hypothesis was based on computational analysis of cMDH primary sequence by hydropathy analysis and the program TM-Pred (http://www.ch.embnet.org), which predicts transmembrane domains. Studies are currently underway that use a variety of methods to determine whether cMDH contains transmembrane domains. The current studies provide the first clear evidence that cMDH is localized to the plasma membrane in LLC-PK_1 cells. Thus, cMDH is found at a location within the cell that is consistent with its role as a regulator of NACh activity.

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