Molecular Cloning and Characterization of p64, a Chloride Channel Protein from Kidney Microsomes*

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Chloride channels were previously purified from bovine kidney cortex membranes using a drug affinity column. Reconstitution of the purified proteins into artificial liposomes and planar bilayers yielded chloride channels. A 64-kDa protein, p64, identified as a component of this chloride channel was used to generate antibodies which depleted solubilized kidney membranes of all chloride channel activity. This antibody has now been used to identify a clone, H2B, from a kidney cDNA library. Antibodies, affinity-purified against the fusion protein of H2B also depleted solubilized kidney cortex from all chloride channel activity. The predicted amino acid sequence of p64 shows that it contains two and possibly four putative transmembrane domains and potential phosphorylation sites by protein kinase A, protein kinase C, and casein kinase II. There was no significant homology to other protein (or DNA) sequences in the data base. The protein is expressed in all cells tested. Expression of its mRNA in Xenopus laevis oocytes led to the insertion of a protein with the appropriate molecular mass in microsomes but not in the plasma membrane. It is likely that p64 represents the chloride channel of intracellular organelles.

Chloride channels are present in the plasma membranes of neurons, fibroblasts, lymphocytes, muscle, and epithelia (1-4). In these cells they are important for mediating a variety of functions, including control of the membrane potential and the regulation of transepithelial ion absorption and secretion. Chloride channels are also present in some intracellular organelles, such as Golgi and endocytic vesicles where they are frequently present in parallel to a proton-translocating ATPase and serve to regulate the pH of these organelles (5-10). Based on single channel behavior, chloride channels exhibit marked diversity in conductance, current-voltage relation, and regulation by modulators. There is probably no cell without one or another type of chloride channel.

The sequences of four chloride channels have recently become available. The neuronal ligand-gated chloride channel (11) is structurally homologous to the nicotinic acetylcholine receptor. A voltage-gated chloride channel from Torpedo electric organ was recently identified by expression cloning (12), and a homologue (CIC-1) was found in mammalian skeletal muscle (13). More recently another homologue (CIC-2) was found to be ubiquitously expressed (14). This family of proteins have at least 12 putative transmembrane domains and cause the expression of voltage-gated chloride channels in Xenopus oocytes. A kidney chloride channel was identified from Madin-Darby canine kidney cells which leads to the expression of a nucleotide-regulated channel whose sequence does not display the typical hydrophobic helices associated with membrane proteins (15). Finally, introduction of the cystic fibrosis gene product, the cystic fibrosis transmembrane conductance regulator (CFTR) (16), into heterologous cells leads to expression of cyclic AMP-activated chloride channels (17, 18). Furthermore, when the positive charges in the first or sixth transmembrane domain were eliminated, the anion selectivity was altered (19). Reconstitution of overexpressed and purified CFTR into planar bilayers resulted in a chloride channel with similar characteristics to the cAMP-regulated channels found in native epithelia, suggesting that CFTR itself is a chloride channel (20).

In contrast to the genetic studies mentioned above, a biochemical approach to the characterization of chloride channels has implicated two additional proteins. Ran and Benos (21) purified and reconstituted a 38-kDa protein from bovine trachea. We tested the indanyloxyacetic acids (IAA) as inhibitory ligands for epithelial chloride channels (22) and identified one of them, IAA-94, as a ligand that had an inhibitory and binding potency in the micromolar range. We purified IAA-binding proteins from bovine kidney cortex using an IAA affinity column and demonstrated that the purified material contained chloride channel activity by two independent criteria (23). First, we showed that purified IAA-binding proteins when incorporated into liposomes supported voltage-sensitive chloride uptake. Furthermore, when these liposomes were fused with planar lipid bilayers, single chloride channels were observed. The material purified by IAA columns consisted of four major proteins, and one, a 64-kDa protein (p64), elicited a monospecific antiserum which immunodepleted all recon-

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EMBL Data Bank with accession number(s) L8547.

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The abbreviations used are: CFTR, cystic fibrosis transmembrane conductance regulator; IAA, indanyloxyacetic acid; PAGE, polyacrylamide gel electrophoresis; PBS, phosphate-buffered saline; RIPA, radioimmunoprecipitation buffer; kb, kilobase(s); CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid.

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stitable chloride channel activity from solubilized bovine renal cortex membranes, suggesting that p64 is a component of a kidney chloride channel (24). Using immunoblots, the anti-p64 sera recognized proteins with similar apparent molecular masses in a variety of epithelial and non-epithelial cells from different species. In addition, this antibody stained the apical membrane and intracellular organelles of epithelial cells, suggesting that p64 may be related to some plasma membrane and vacuolar chloride channels. The two most abundant proteins purified by the IAA affinity column (molecular masses, 100 and 27 kDa) were identified by NH2-terminal sequence to be cellular proteins that are known to bind to ethacrynic acid, the parent structure of IAA (24). Hence, we concluded that they were drug-binding proteins which were enriched by IAA affinity chromatography but were unrelated to the chloride channel.

Recent studies by other investigators support the hypothesis that a protein of approximately 64 kDa may be a component of a vacuolar chloride channel. Brain clathrin-coated vesicles and kidney endosomes were isolated by precipitation by opening a chloride channel and phosphorylating a protein of similar molecular mass (10, 25, 26). These results suggest that p64, which we have purified, might be a chloride channel of intracellular organelles. We report here the primary sequence of p64.

EXPERIMENTAL PROCEDURES

Identification of H2B and Sequence Analysis—All RNA samples were prepared from quick-frozen slaughterhouse specimens or cultured cells by the method of Chomczynski and Sacchi (27). Oligo(dT)-cellulose enrichment of poly(A) RNA was carried out by standard methods (28). Oligo(dT) random-primed cDNA was synthesized from bovine kidney cortex poly(A) RNA using the Klenow fragment of DNA polymerase, cut with BamHI, ligated with pBluescript (Stratagene, La Jolla, CA), and prepared with the cDNA insert from plasmid H2B, labeled with [α-32P]dCTP using the random priming kit from Boehringer Mannheim. The bovine samples were probed at high stringency with the final was in 0.1 × SSC, 0.1% SDS at 60 °C for 30 min. The human and shark samples were probed at somewhat lower stringency with a final wash in 0.1 × SSC, 0.1% SDS at 55 °C for 15 min.

Polymerase Chain Reaction of Anti-p64 Antisera—Guinea pig antisera were prepared against gel slices of 64-kDa proteins purified on an IAA-23 column. These antisera, termed anti-p64, were found to immunodeplete chloride channel activity (24). The anti-p64 serum was affinity-purified to generate an anti-H2B antibody and used for Western blot and immunodepletion experiments. E. coli lysates expressing the H2B insert were subjected to preparative SDS-PAGE and transferred to nitrocellulose. After blocking with 5% milk, the blots were incubated with anti-p64 antisera preabsorbed against E. coli lysates lacking the insert. The bound antibody was visualized using alkaline phosphatase-linked goat anti-rabbit antibodies to probe a vertical strip of the nitrocellulose. The bound antibody was eluted from the indicated region of the remaining nitrocellulose using 0.2 M glycine, pH 2.3. Control sera (non-H2B) were obtained by identical elution of anti-p64 antisera from preparative SDS-PAGE gels of proteins from E. coli transfected with vectors lacking the H2B insert at the same molecular mass region as H2B.

For Western blot, kidney cortex vesicles were solubilized with n-octyl glucoside and separated on a Sephadex HR200 gel filtration column (2.5 × 90 cm). The fraction containing reconstitutable 32Cl uptake activity was subjected to SDS-PAGE and immunoblot assay with anti-H2B.

H2B was cloned into an expression vector pE2X, and a laclZ-p64 fusion protein was generated in the bacterial host N4830-a (33). The fusion protein was constructed by inserting the large PstI fragment of H2B into the unique PstI site of the parent plasmid. Antibodies were raised in rabbits by immunizing with the partially purified fusion protein.

Reconstitution of Chloride Channels and Immunodepletion of Activity—Solubilized kidney membranes (3 mg/ml) were incubated for 18 h at 4 °C with anti-H2B (i.e. anti-p64 antibodies affinity-purified from baculovirus-infected Sf9 cells) and a lactZ-H2B (anti-p64 affinity-purified against the same region of E. coli protein) affinity-antibody-protein complex, resulting in the complete abolition of 32Cl uptake activity.
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35Cl uptake assays were performed as described previously using the method of Garty (22, 34). In brief, external chloride was removed by applying the vesicles to a glucosolate-exchange column and eluting with 250 mM sucrose (22). 5 × 10⁶ cpm/ml of 35Cl was added. Immediately half the sample was removed and valinomycin added to a final concentration of 5 μM. At the indicated time points 0.5-ml samples were taken and extravesicular 35Cl removed by passing the vesicles through an 8-cm anion exchange column. 35Cl uptake was then measured by liquid scintillation counting.

**Electrophysiology of Xenopus laevis Oocytes**—Oocytes were always completely denuded of follicular epithelium by treating them for 1-2.5 h with collagenase followed by incubation in hypertonic K asparagine for 45 min. p64 mRNA was injected and the oocytes were assayed 3, 6, or 7 days after injection. Changes in membrane conductance were assayed by measuring the current changes in response to voltage pulses using a standard 2-microelectrode voltage clamp set at a holding potential of −100 mV. The maximum change in current was recorded in response to 50 μM cytosolic AMP for determination of GoAMP, or 10 μM bath A23187 for GcAMP. Incubations were at 18 °C, and recordings were made at room temperature (21–25 °C). The bath contained (in mM) 90.4 sodium, 1 potassium, 0.74 calcium, 0.82 magnesium, 89.8 chloride, 2.4 HCO₃, 0.82 SO₄, 0.66 NO₃, and 1 HEPES (titrated to pH 7.4 with NaOH). Also included were penicillin 100 U/ml, streptomycin (100 μg/ml), and gentamycin (50 μg/ml).

**Preparation of Membrane Vesicles from Xenopus Oocytes**—Intracellular vesicles were isolated by minor modifications of the method of Evans and Kay (35). 40–50 oocytes were washed with ice-cold homogenization buffer (50 mM NaCl, 10 mM MgCl₂, 20 mM Tris-HCl, pH 7.6, 1 mM phenylmethanesulfonyl fluoride, and 1 mM leupeptin) and homogenized in a loose-fitting glass homogenizer with 10 strokes using 10–20 μl of buffer/oocyte. The homogenate was layered on a 400-μl cushion of 20% sucrose in homogenizing buffer and spun for 30 min in a table top Microfuge at the maximum speed. The supernatant, now comprising the vesicle fraction was then centrifuged in the Microfuge at top speed. The supernatant, composed largely of the cytosol, was discarded and the pellet was rehomogenized in 200 μl of phosphate-buffered saline (PBS) containing 1% Nonidet P-40 and 1 mM phenylmethanesulfon fluoride using 10 strokes and spun for 10 min in the Microfuge at top speed. The supernatant, now comprising the vesicle fraction was then analyzed by SDS-PAGE and immunoblotting methods.

**Biotinylation** (Ref. 36)—Cells were washed twice with PBS containing calcium and magnesium and incubated in PBS containing 0.5 mg/ml NHS-SS-biotin (Pierce Chemical Co.) at 4 °C for 30 min while slowly shaking. The reaction was stopped by washing twice with 0.5 M Tris, pH 8.0. The cells were then solubilized in RIPA buffer (1 ml/l T-75 plate or 20 oocytes) and the nonsolubilized material separated by centrifugation in the Microfuge at top speed for 30 min. The supernatant was added to 50 μl of streptavidin-conjugated Sepharose beads (1 μg/ml). After a 30-min incubation at 4 °C with gentle shaking, the beads were washed four times with RIPA buffer containing 0.5 M NaCl followed by two washes with RIPA buffer. The beads were then mixed with 50 μl of SDS-PAGE sample buffer and boiled for 3 min to release the disulfide-linked proteins from the beads which were then removed by centrifugation. The eluted proteins were then subjected to SDS-PAGE and electrophoretically followed by immunoblotting using anti-p64 antibodies.

**RESULTS**

**Cloning and Sequencing of p64**—We had previously generated an antibody against a 64-Da protein that was purified using an IAA-23 column. This antibody, termed anti-p64 immunodepleted chloride channel activity from solubilized bovine renal cortex membranes (24). We constructed a cDNA expression library from bovine renal cortex membranes, which has been transfected with the bovine renal proteins. We were then solubilized and the supernatant proteins were expressed in pBluescript; approximately 63 kDa is predicted to be contributed by the cDNA insert. To assess the identity of this clone, anti-p64 antiserum was affinity-purified against the overexpressed H2B fusion protein, and the resulting fraction was termed anti-H2B. As a control antiserum, we "affinity-purified" the same anti-p64 serum against the 70-kDa region of E. coli proteins. These two affinity-purified antibodies were used in two assays. First, on a blot of proteins from bovine renal cortex membranes, anti-H2B antiserum stained a 64-kDa protein just as did the parent antibody prepared against he purified kidney protein (Fig. 1B). Anti-p64 sera recognize the H2B fusion protein, and anti-H2B antibodies recognize p64, A, immunoblot of E. coli transformed with H2B clone or clone with H2B or with the parent plasmid with no insert probed with a 1:1000 dilution of anti-p64 antiserum. B, immunoblot of partially purified bovine kidney cortex membranes probed with affinity-purified anti-H2B. Preparation of antibodies is described under "Experimental Procedures."

**Fig. 1. Anti-p64 sera recognize the H2B fusion protein, and anti-H2B antibodies recognize p64.** A, immunoblot of E. coli transformed with H2B clone or clone with H2B or with the parent plasmid with no insert probed with a 1:1000 dilution of anti-p64 antiserum. B, immunoblot of partially purified bovine kidney cortex membranes probed with affinity-purified anti-H2B. Preparation of antibodies is described under "Experimental Procedures."

**Fig. 2. Depletion of chloride channel activity by anti-p64 antibody affinity-purified against clone H2B fusion protein.** Anti-p64 antiserum was affinity-purified against the H2B fusion protein (circles) or against the same region of an E. coli lysate which was transformed with a vector without an insert (triangles). Bovine kidney cortex microsomes were solubilized and incubated with the two batches of sera; the antigen-antibody complexes were then precipitated with protein A beads and the supernatant proteins reconstituted as described under "Experimental Procedures." 35Cl uptake was then measured in the presence (open symbols) or absence (closed symbols) of valinomycin to collapse the membrane potential. Voltage-sensitive 35Cl transport is the difference between the two curves. The results shown are the average of three independent experiments.
The recombinant cDNAs used for expression where the internal deletions are marked. Nonsense codons occur in all reading frames. The predicted amino acid sequence is shown below the nucleotide sequence, and positions are numbered on the right. The three bottom clones represent hydrophobicity plot.

**FIG. 3.** Complementary DNA and predicted amino acid sequence of p64. A, a schematic structure for p64 cDNA. The top line represents the full-length cDNA; the predicted open reading frame is indicated by the black box. Individual cDNAs used to determine the sequence are represented below. The three bottoms clones represent the full-length cDNA; the predicted open reading frame is immediately following the second PstI site in pNH. Restriction enzymes shown are C, ClaI; E, EcoRI; H, HincII; P, PstI. B, nucleotide sequence of bovine p64 with nucleotide positions denoted on the left. The predicted amino acid sequence is shown below the nucleotide sequence, and positions are numbered on the right. C, Kyte-Doolittle hydrophobicity plot.

It is necessary to use this experimental paradigm, since reconstitution will dilute the chloride channel protein such that only a small fraction of the vesicles will contain a chloride channel. Hence, to obtain a significant signal, it is necessary to choose conditions that will result in accumulation of the tracer well above the equilibrium level.

3Cl uptake in liposomes is complicated also by the fact that lipid vesicles have an inherent chloride-chloride exchange activity (37). Hence, there will be a finite rate of uptake into all liposomes. Because the number of liposomes that do not contain a chloride channel.
contain channels is larger than those that do, the background uptake is of sufficient magnitude to make attempts at measurement of only initial rates futile. Hence, the uptake with the greatest specificity for channel-mediated transport is that affected by collapsing the membrane potential by valinomycin.

When solubilized vesicles were treated with anti-H2B antibodies, they were unable to mediate valinomycin sensitive $^{36}Cl$ uptake (Fig. 2). Solubilized membranes incubated with the same sera affinity-purified against E. coli proteins lacking H2B were able to mediate valinomycin-sensitive transport. From these results we conclude that H2B is a necessary component of the kidney cortex chloride channel.

H2B was used to screen the original oligo(dT)-primed library, and a subclone of the 5' end was used to screen a randomly primed library. Overlapping clones were obtained and sequenced (Fig. 3). The 5' end of the message was obtained with anchored polymerase chain reaction using nested oligonucleotide primers. A partial restriction map of the cDNA clones is shown in Fig. 3A. The sequence of the entire coding region was obtained from at least two independently derived cDNA clones.

The full-length cDNA is 6160 nucleotides (Fig. 2B). The first methionine codon is at nucleotide 157 and is preceded by a stop codon at position 60 in the same reading frame. The sequence around the first ATG conforms with the Kozak sequences for translation initiation sites (38). The first stop codon occurs at nucleotide 1468, resulting in an open reading frame coding for a protein of 437 amino acids. There is an extremely long 3'-untranslated region which ends in a poly(A) tail preceded by a typical polyadenylation sequence at nucleotide 6116. There is no extended open reading frame in the 3'-translated region.

The predicted translation product has a calculated molecular mass of 49,008 daltons. To confirm the identity of the deduced translational initiation and termination sites, a construct named pNH containing the entire coding region downstream from a T7 viral promoter was assembled from individual cDNAs and modified in one of two manners (Fig. 3A). Translational stop codons were introduced at nucleotide 1516 (numbered according to the full-length sequence), 48 nucleotides downstream of the endogenous termination codon in plasmid pNH and at position 1294 within the open reading frame in plasmid pNHAP. The parent plasmid and each construct with introduced termination codons were used as templates in an in vitro transcription/translation assay using T7 RNA polymerase and a reticulocyte lysate (Fig. 4). Both pNH and pNHAP with predicted translation products of 49,008 Da yielded products with an identical apparent molecular mass of 44 kDa on SDS-PAGE. pNHAP with a predicted translation product of 42,306 daltons yielded a product with an apparent molecular mass of 58 kDa. Thus we conclude that the predicted initiation and termination codons are correctly identified and that p64 has aberrant mobility on SDS-PAGE.

Neither the nucleotide nor the predicted amino acid sequence of p64 bear significant homology to any known gene or gene product. The deduced amino acid sequence is markedly rich in acidic residues and has a predicted pI of 4.14. A hydrophobicity analysis of the predicted amino acid sequence by the method of Kyte and Doolittle (39) is shown in Fig. 3C. There are at least two potential transmembrane domains at amino acids 201-236 and 367-385. No putative signal sequence was discerned, suggesting that the NH$_2$ terminus of the protein would be predicted to be in the cytoplasm. This large amino terminus section of the protein contains consen-

![Fig. 4. Cell-free translation of p64 mRNA.][1]

The reticulocyte lysate was assayed with transcripts from the following plasmids, pNH, which contains the naturally occurring sequences from 1 to 2500 in the full-length message; pNHAP, stop codon engineered immediately after the endogenous stop codon; pNHAP, stop codon engineered 5' to endogenous stop codon.

**Expression of p64 in Different Cell Types**—Using antibodies raised against an H2B fusion protein, we identified proteins of apparent molecular mass of 64 ± 5 kDa in all cell types tested, namely, human colon carcinoma cell line T84, a human pancreatic adenocarcinoma cell line obtained from a patient with cystic fibrosis, CFPAC, and a cell line that had been rescued by transfection with the wild type CFTR gene, a mouse fibroblast cell line, 3T3, and the Sf9 insect ovary cell line (Fig. 5). In the epithelial cell lines there were additional bands at a molecular mass of 90 kDa and occasionally 120 kDa (in the case of T84) that reacted with this antibody. The

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[1]: https://example.com/f4.png
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Preimmune Immune

FIG. 5. Immunoblot with an antibody raised against an H2B fusion protein. A crude cell membrane fraction was generated after homogenization of different cell lines and discarding the nuclear pellets. These membrane fractions were extracted with 1 M KSCN, and the 100,000 × g pellets were used in the immunoblot.

RNA from chloride-transporting epithelia were also probed with p64 cDNA as shown in Fig. 6. T84 cells and Panc 1 are continuous human cell lines which have well characterized chloride channels. Both of these cell lines have a single major transcript of approximately 5.5 kb which hybridizes to the p64 probe at high stringency. RNA from the shark rectal gland, a chloride-secreting epithelium rich in chloride channels, contains transcripts of approximately 4 and 6 kb that hybridize with the p64 probe at moderate stringency. Thus, at the protein and RNA level, p64 was present in every cell type that was tested.

Expression of p64 and Chloride Channels—We injected RNA transcribed from the T7 promoter construct of the full-length clone into Xenopus oocytes. No new plasma membrane chloride current appeared on electrophysiological studies. However, a new protein with the appropriate molecular mass appeared. Cell fractionation experiments showed that the protein was incorporated into microsomes (Fig. 7). However, the protein was unable to be targeted to the plasma membrane. We biotinylated the oocytes and precipitated the solubilized proteins by avidin beads. Western blot analysis showed that p64 was not present in the precipitate (Fig. 7). A similar experiment performed on CFPAC and T84 cells, where we had shown previously that this protein reaches the plasma membrane, demonstrated that this method is capable of documenting the presence of p64 (data not shown). The reason why expressed p64 cannot reach the surface is not apparent at present, but it could be due to the need for another protein or that it could contain a yet unidentified retention signal for intracellular organelles.

DISCUSSION

We have presented the cloning and characterization of a gene encoding a component of bovine kidney cortex chloride channels. The protein predicted to be encoded by this gene has a sequence consistent with an integral membrane protein which crosses the membrane at least twice and has target sequences within the proposed cytoplasmic domains for several signal transduction pathways known to affect chloride channel activity in epithelial cells. This gene is expressed to some extent in all cells tested so far. In addition it is expressed...
bilayers showed single channels, but since this is a single primary antibody was used. 

quantitative assay that allows an order of magnitude calculation of yield. Using this assay we determined previously that the conductance activity despite attempts at renaturation using the method of Braiman et al. (42). This has prevented us from examining directly whether p64 alone can reconstitute chloride channels. Hence, at the minimum, p64 is a necessary component of the kidney cortex chloride channel complex. The availability of the cDNA should allow us now to overexpress the protein to obtain sufficient quantities for further biochemical and functional studies similar to what was done with CFTR (20).

Is p64 a Vacuolar Chloride Channel?—p64 was purified from microsomal membranes enriched for Golgi markers. It is present in all cells, albeit at low abundance. In some cells its abundance is high enough to allow immunocytochemical localization. This was achieved in CFPAC cells (a pancreatic duct adenocarcinoma cell obtained from a patient with cystic fibrosis (43)) which showed that perinuclear vesicles were stained (24). There is increasing evidence that the chloride channels of endosomes and clathrin-coated vesicles can be activated by protein kinase A (10, 25). Furthermore, such phosphorylation caused the appearance of a phosphorylated 65–70-kDa protein (25, 26). All of this leads one to suspect that p64 could be the chloride channel of intracellular membranes.

When p64 was expressed in Xenopus oocytes, it was incorporated into membranes but was not targeted to the plasma membrane. Whether the protein has signals that retain it in intracellular organelles or lacks signals that carry it to the plasma membrane remains to be determined. We demonstrated previously that p64 was present on the apical membrane of some CFPAC cells. Whether this is due to the presence of another protein that can guide it to that domain or that the CFPAC cell expresses a homologue of p64 that has a targeting signal remains to be determined. As shown in the Northern blots there are several homologous messages of p64 in the kidney, suggesting that such diversity of structure might underlie diverse fates of p64.

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