Functional expression and apical localization of the cystic fibrosis transmembrane conductance regulator in MDCK I cells

Abdalla MOHAMED*, Donald FERGUSON*, Fabian S. SEIBERT*, Hou-ming CAI†, Norbert KARTNER†, Sergio GRINSTEIN*, John R. RIORDAN§ and Gergely L. LUKACS*‡||

*The Hospital for Sick Children, Research Institute, 555 University Avenue, Toronto M5G 1X8, Canada, †Department of Pharmacology and ‡Department of Clinical Biochemistry, University of Toronto, Toronto M5S 1A8, Canada, and §S. C. Johnson Medical Research Center, Mayo Clinic, Scottsdale, AZ 85259, U.S.A.

The gene product affected in cystic fibrosis, the cystic fibrosis transmembrane conductance regulator (CFTR), is a chloride-selective ion channel that is regulated by cAMP-dependent protein kinase-mediated phosphorylation, ATP binding and ATP hydrolysis. Mutations in the CFTR gene may result in cystic fibrosis characterized by severe pathology (e.g. recurrent pulmonary infection, male infertility and pancreatic insufficiency) involving organs expressing the CFTR. Interestingly, in the kidney, where expression of the CFTR has been reported, impaired ion transport in patients suffering from cystic fibrosis could not be observed. To understand the role of the CFTR in chloride transport in the kidney, we attempted to identify an epithelial cell line that can serve as a model. We demonstrate that the CFTR is expressed constitutively in Madine–Darby canine kidney (MDCK) type I cells, which are thought to have originated from the distal tubule of the dog nephron. We show expression at the mRNA level, using reverse transcriptase-PCR, and at the protein level, using Western blot analysis with three different

monoclonal antibodies. Iodide efflux measurements indicate that CFTR expression confers a plasma membrane anion conductance that is responsive to stimulation by cAMP. The cAMP-stimulated iodide release is sensitive to glybenclamide, diphenylamine carboxylic acid and 5-nitro-2-(3-phenylpropylamino)benzoic acid, but not to 4,4'-di-isothiocyanostilbene-2,2'-disulphonic acid, an inhibitor profile characteristic of the CFTR chloride channel. Finally, the polarized localization of the CFTR to the apical plasma membrane was established by iodide efflux measurements and cell-surface biotinylation on MDCK I monolayers. Interestingly, MDCK type II cells, which are thought to have originated from the proximal tubule of the kidney, lack CFTR protein expression and cAMP-stimulated chloride conductance. In conclusion, we propose that MDCK type I and II cells can serve as convenient model systems to study the physiological role and differential expression of CFTR in the distal and proximal tubule respectively.

INTRODUCTION

Cystic fibrosis (CF) is one of the the most frequently occurring recessive genetic disorders, affecting approx. 1 in 2500 live births in Caucasian populations. The disease is characterized by abnormalities in transepithelial electrolyte flow with subsequent pathology of many organs, including the lung, the pancreas and the reproductive system [1–3]. The CF gene encodes an integral membrane glycoprotein, the cystic fibrosis transmembrane conductance regulator (CFTR), which belongs to the ABC transporter superfamily and functions as a chloride channel, regulated through phosphorylation by cAMP-dependent protein kinase (PKA) and ATP hydrolysis [1,2,4–8]. To date, more than 500 CF mutations have been described [9,10]. Individual mutations may lead to impaired protein synthesis, abnormal processing of the CFTR or defective regulation of the CFTR chloride channel, or a combination of these abnormalities [6–10].

The expression of the CF gene, which is subject to both hormonal and developmental regulation, has been analysed by immunohistochemistry, Northern blot analysis and *in situ* hybridization in several organs, including the lung, sweat glands, pancreatic duct, reproductive organs and heart [11–16]. Ex-

pression of the CFTR in human cell lines, most of them of epithelial origin, varies from very low to relatively high levels [12,17–19]. In some instances, although the cAMP-activated chloride current characteristic of the CFTR was documented, detection of the CFTR by immunological techniques was unsuccessful [20,21].

The role of the CFTR in the kidney is controversial. Although CFTR expression was reported at both the mRNA and protein levels, impaired chloride transport in the kidney has not been noted in CF [2,22,23]. On the other hand, recent patch-clamp data are consistent with the notion that the CFTR may play a role in transepithelial chloride movements in the distal tubule. In primary cells of kidney medullary and cortical collecting ducts, a cAMP-stimulated chloride current, with characteristics similar to those of the CFTR, was documented and proposed to participate in chloride secretion and/or reabsorption [24–26].

In an attempt to identify a kidney-derived epithelial cell line that could serve as a convenient model with which to investigate the role of the CFTR, we discovered that Madine–Darby canine kidney (MDCK) type I cells, one of the most thoroughly characterized, highly differentiated epithelial cell lines, express the CFTR. This conclusion was reached by using molecular

Abbreviations used: BHK, baby hamster kidney; CF, cystic fibrosis; CFTR, cystic fibrosis transmembrane conductance regulator; CHO, Chinese hamster ovary; DPC, diphenylamine carboxylic acid; DIDS, 4,4'-di-isothiocyanostilbene-2,2'-disulphonic acid; IBMX, 3-isobutylmethyl-1-xanthine; NPPB, 5-nitro-2-(3-phenylpropylamino)benzoic acid; MDCK, Madine–Darby canine kidney; NHS-SS-biotin, sulphosuccinimido-biotin; PKA, cyclic AMP-dependent protein kinase; RT-PCR, reverse transcriptase-PCR.

|| To whom correspondence should be addressed, at The Hospital for Sick Children.

biological, biochemical, functional and pharmacological approaches. In addition, association of the CFTR with the apical plasma membrane was demonstrated by polarized iodide efflux measurements and cell-surface biotinylation of MDCK I monolayers, cultured on a permeable support.

MATERIALS AND METHODS

Materials

Forskolin, 8-(4-chlorophenylthio)-cAMP, glybenclamide, 4,4'-di-isothiocyanostilbene-2,2'-disulphonic acid (DIDS) and 3-isobutylmethyl-1-xanthine (IBMX) were purchased from Sigma. Diphenylamine carboxylic acid (DPC) was obtained from Fluka, and 5-nitro-2-(3-phenylpropylamino)benzoic acid (NPPB) was a gift from Dr. A. Edelman (INSERM Unit 323, Paris, France).

Cell culture

MDCK type I cells (A.T.C.C. accession no. CCL34), T84 cells (A.T.C.C. accession no. CCL248), a sweat duct reabsorptive cell line (RD2; kindly provided by Dr. C. Bell and Dr. P. Quinton, University of California, Riverside, CA, U.S.A.) and MDCK II cells (kindly provided by Dr. S. Hansen, Harvard University, Boston, MA, U.S.A.) were maintained in a 1:1 (v/v) mixture of Dulbecco's modified Eagle's medium and F-12 (Ham) medium supplemented with 5% (v/v) fetal bovine serum, 50 units/ml penicillin and 50 µg/ml streptomycin (Gibco BRL Life Technologies, Inc., Gaithersburg, MD, U.S.A.). PANC-1 (A.T.C.C. accession no. CRL1469), a pancreatic duct cell line, was cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, 50 units/ml penicillin and 50 µg/ml streptomycin. CaCo-2, HT-29 and CFTR-expressing Chinese hamster ovary (CHO) cells were cultured as described [27]. All cells were grown at 37 °C in 5% CO₂ on treated plastic tissue culture dishes. When MDCK I and II cells were cultured on Costar polycarbonate permeable filter supports (0.4 µm pore size), cells were seeded at confluent density and grown for an additional period of 2–7 days. Medium was replaced in both the apical and basolateral compartments every 2 days.

Reverse transcriptase (RT)-PCR

Total RNA was isolated from the various cell lines using a protocol described previously [28]. Denatured RNA (1 µg) was annealed to random hexamers (1.5 mM; Pharmacia) or to oligo(dT)₁₅ and subsequently converted into complementary DNA using avian myeloblastosis virus reverse transcriptase (1.2 units; Promega) or SuperScript II RNase H⁻ reverse transcriptase (200 units; Gibco/BRL). Since the nucleotide sequence of the canine CFTR is not available and the primary sequence identity between mammalian CFTRs is high, our primer design was based on the human CFTR sequence. The forward and reverse primers used for amplification corresponded to nucleotides 3907–3924 (5'-AGACTACTGAACACTGAA-3') and 4629–4647 (5'-TTCCATGAGGTGACTGTCC-3') respectively of the human CFTR cDNA. To verify the specificity of the RT-PCR product, a second primer pair was utilized, comprising nucleotides 2481–2512 (5'-TCACCGAAAGACAACAGCATCCACACGAAAAG-3') and 2747–2777 (5'-CACAGCACAACCAAGAGCAGCCACCTC-3') of the human CFTR cDNA. To normalize for total RNA, a primer pair for human β-actin was included (upstream primer, nucleotides 375–399; downstream primer, nucleotides 572–593) in some reactions. For the first and second sets of CFTR primers, the PCR reactions were carried out for 30 cycles at 94 °C for 30 s, 50 °C for 1 min and 72 °C for 2 min, and for 30 cycles at 94 °C for 40 s, 60 °C for 1 min and

72 °C for 2 min, respectively. DNA fragments were visualized by staining the agarose gels with ethidium bromide. The following negative controls were included: water added instead of RNA, no reverse transcriptase included, and RNA extracted from CHO or baby hamster kidney (BHK) cells lacking CFTR expression.

Immunoblot analysis of CFTR expression

Confluent monolayers (2–7 days post-confluence) were washed with ice-cold PBS and lysed in RIPA buffer (25 mM Tris, 150 mM NaCl, 0.5% deoxycholate, 0.1% SDS, 0.1% Triton X-100, pH 8.0). Nuclei and unbroken cells were removed by centrifugation (15000 g for 15 min at 4 °C). Soluble proteins in the supernatant were denatured with Laemmli sample buffer, fractionated on SDS/7%-PAGE gels and transferred to a nitrocellulose membrane. Transferred proteins were probed with the monoclonal anti-CFTR antibodies M3A7, L12B4 [12] and 24-1 (Genzyme; [29]). M3A7 and L12B4 are known to recognize human, rat, mouse and bovine CFTRs ([12]; N. Kartner and J. Riordan, unpublished work), and the 24-1 monoclonal antibody is known to recognize human, monkey and *Xenopus* CFTRs (J. Marshall, personal communication). The primary antibody was visualized by using horseradish peroxidase-conjugated sheep anti-mouse immunoglobulin and the enhanced chemiluminescence (ECL) Western Blot Kit (Amersham), as previously described [30].

Domain-selective cell-surface biotinylation of MDCK monolayers

MDCK cells were grown to confluency on polycarbonate filters (Costar; 0.4 µm pore size). Tight-junction formation was determined by measuring the transepithelial electrical resistance either with an Endohm tissue culture chamber or with electrodes connected to a Millicell resistance meter. The electrical resistance of confluent monolayers (2–7 days post-confluence) of MDCK I and MDCK II cells was 500–800 and 100–180 Ω·cm² respectively. Tight monolayers of MDCK I cells were labelled from the apical or the basolateral surface with the membrane-impermeant agent sulphosuccinimido-biotin (NHS-SS-biotin; Pierce; 1 mg/ml) on ice [31,32]. Following extensive washing with ice-cold PBS, cells were lysed in RIPA buffer containing a protease inhibitor cocktail (10 µg/ml each of leupeptin, pepstatin, chymostatin and antipain, 10 mM iodoacetamide and 1 mM PMSF). Biotinylated proteins were affinity-purified on a monomeric avidin column [32] and eluted with 1 mM biotin. Subsequently, the biotinylated CFTR was isolated by immunoprecipitation with the M3A7 and L12B4 monoclonal antibodies [12,30]. To detect the relatively low amount of biotinylated CFTR, the CFTR was phosphorylated *in vitro* in the presence of the catalytic subunit of PKA (Promega) and [γ -³²P]ATP, and visualized by autoradiography [33,34].

Iodide efflux measurements

Iodide efflux was performed essentially using the protocol described for CFTR-transfected CHO monolayers [34]. In brief, the chloride content of MDCK cells was replaced with iodide by incubating the cells in loading buffer [136 mM NaI, 3 mM KNO₃, 2 mM Ca(NO₃)₂, 11 mM glucose, 20 mM Hepes, pH 7.4] for 60 min at room temperature. Iodide efflux was initiated by replacing the loading buffer with efflux medium (composed of 136 mM nitrate in place of iodide). To obtain a time course of iodide efflux, the extracellular medium was replaced every 1 min with efflux buffer (1 ml). After the iodide release had reached a steady state (usually 3–5 min), the intracellular cAMP level was raised by agonists (10–20 µM forskolin, 0.5 mM dibutyryl-cAMP

and 0.2 mM IBMX, or 20 μ M forskolin alone) and collection of the efflux medium was resumed for an additional 6–9 min. In some experiments dibutyryl-cAMP was replaced with 0.5 mM 8-(4-chlorophenylthio)-cAMP. The amount of iodide in each sample was determined with an iodide-selective electrode [34].

The iodide-permeability of the apical and basolateral plasma membrane was assessed on tight monolayers seeded on permeable polycarbonate filter supports. Following the accumulation of iodide in confluent MDCK I and MDCK II monolayers, iodide release into both the apical and basolateral compartments was monitored as described above.

RESULTS

Detection of CFTR transcripts in MDCK cells by RT-PCR

The level of expression of the CFTR in native epithelia and immortalized cell lines is relatively low, and transcripts cannot be

Figure 1 RT-PCR analysis of CFTR expression in MDCK I and II cells and other epithelia

All cells were grown for 2 days post-confluence on plastic supports. **(A)** Transcription of CFTR mRNA in MDCK I cells. Samples of 1.0 μ g of total RNA from a confluent monolayer were reverse-transcribed to cDNA and PCR-amplified according to the protocol described in the Materials and methods section. Lane 1, RT-PCR product of total cellular RNA in the absence of reverse transcriptase; lane 2, RT-PCR product of the same RNA in the presence of reverse transcriptase; lane 3, RT-PCR product from 50 ng of pBluescript-CFTR (cDNA); lane 4, 1 kb DNA ladder. **(B)** RT-PCR analysis of CFTR transcripts in various epithelia. RT-PCR was performed as described in the Materials and methods section from 1.0 μ g total RNA extracts of RD2, PANC-1 and T84 cells (lanes 2, 3 and 4 respectively). No RT-PCR product was detectable in the absence of reverse transcriptase (lane 1). A 1 kb DNA ladder is shown in lane 5. **(C)** RT-PCR results from HT-29 (lane 2), MDCK I (lane 3) and MDCK II (lane 4) cells. For the RT-PCR reaction demonstrated in **(A)** and **(B)**, the primer set used amplified the C-terminal sequence of CFTR. In **(C)** the RT-PCR reaction comprised the primer set corresponding to the exon 13/exon 14 junction of the CFTR, along with human β -actin-specific primers. Note the similar amounts of the actin-specific band (~220 bp), in contrast to the marked difference in the amounts of the CFTR-specific band (~290 bp), between samples obtained from HT-29, MDCK I and MDCK II cells **(C)**.

Figure 2 Immunoblot analysis of CFTR expression in MDCK I and MDCK II cells

Solubilized proteins (50–75 μ g) from the indicated cells were fractionated on an SDS/7%-PAGE gel and probed with anti-CFTR antibodies (1.0 μ g/ml). CFTR detection was carried out with the monoclonal antibodies M3A7 and L12B4 [12]. MDCK cells were cultured for 3 days after reaching confluence in the indicated medium (DMEM, Dulbecco's modified Eagle's medium; α MED, minimal essential medium) supplemented with 5–10% (v/v) fetal calf serum.

easily detected by conventional Northern-blot analysis [35–37]. Therefore the more sensitive RT-PCR technique was employed to determine the expression of the CF gene in epithelial and non-epithelial cell lines derived from the kidney. Expression in BHK cells was below the detection limit (results not shown). Earlier reports also failed to demonstrate CFTR transcripts in other cell lines (HEK-293 and LLC-PK) originating from the kidney [37–39]. In contrast, CFTR transcripts could readily be identified in as little as 1 μ g of total RNA extract from MDCK I cells (Figure 1A, lane 2). The transcript size (720 bp) was the same as that obtained by amplification of the CFTR cDNA template cloned into the Bluescript vector (Figure 1A, lane 3). No PCR product appeared when either reverse transcriptase (Figure 1A, lane 1) or the template (results not shown) was omitted.

For comparative purposes, we examined whether CFTR transcripts could be amplified from human cell lines derived from the pancreatic duct (PANC-1) and the reabsorptive sweat duct (RD2). Detection of CFTR mRNA in PANC-1 cells has only been possible when poly(A)⁺ RNA is used as the starting material, despite the fact that, *in situ*, intralobular pancreatic duct cells exhibit some of the highest relative amounts of CFTR so far detected [12,18,40]. A 720 bp PCR product could be visualized by RT-PCR in both RD2 and PANC-1 cells (Figure 1B, lanes 2 and 3), and in T84 cells (lane 4) without poly(A)⁺ selection. A lower-mobility minor transcript was also amplified in PANC-1 cells which may correspond to an alternatively spliced transcript, since we used primers that cover the boundaries of exons 22 and 24. A similar primer selection strategy made it possible to identify an alternatively spliced form of the CFTR (CFTR 24a⁺), which should give rise to a truncated CFTR molecule lacking 61 amino acids at the C-terminus [41].

To provide corroborative evidence for the identity of the RT-PCR product, a second primer pair was used to amplify a CFTR-specific sequence from MDCK cell extracts. This primer pair, which spans the boundary of exon 13 and exon 14, generated a band (293 bp) in MDCK I cells, and to a much lesser extent in MDCK II cells, of precisely the same size as in HT-29, a human positive control cell line (Figure 1C).

Figure 3 Effect of polarization on CFTR expression in MDCK I and MDCK II cells

MDCK I or II cells were seeded on plastic (lanes 1, 2, 4 and 5) or on a permeable filter support (lanes 3 and 6) and cultured for 2 days (lanes 1 and 4) or 7 days (lanes 2, 3, 5 and 6) after reaching confluence. Whole-cell lysates (50 μ g of protein) were fractionated on an SDS/7%-PAGE gel. Proteins were transferred to nitrocellulose and probed with the M3A7 anti-CFTR [12] and 6H anti-(Na/K-ATPase α subunit) antibodies [53]. A whole-cell extract of T84 cells was loaded as a standard (lane 7).

Biochemical identification of the CFTR in MDCK cells

Efficient translation of CFTR mRNA should be manifested in the accumulation of immunoreactive protein and in the presence of cAMP-stimulated anion conductance in MDCK cells. First, CFTR protein expression was assessed by immunoblot analyses of whole-cell extracts of MDCK I and II cells grown on a plastic support, using the monoclonal anti-CFTR antibodies L12B4, M3A7 and 24-1. These antibodies were raised against fusion proteins comprising the first or second nucleotide-binding fold or the C-terminus respectively, and have been extensively characterized [12,29]. All of these antibodies recognized a polypeptide in MDCK I extracts with an apparent molecular mass of 170 kDa (results for M3A7 and L12B4 shown in Figure 2). This common immunoreactive polypeptide corresponds in size to that of the CFTR expressed constitutively in certain epithelia or upon

heterologous expression in polarized or non-polarized cells (Figure 2; and [2,6,7]). Moreover, this immunoreactive band exhibits a heterogeneous electrophoretic mobility, reflected in its diffuse appearance, which is typical of the migration pattern of complex-glycosylated membrane proteins, including the CFTR [33]. Additionally, a minor, faster-migrating band could be seen (Figure 2) which is conceivably the core-glycosylated form of the CFTR [33]. Depending on the medium composition, the level of expression of CFTR protein in MDCK I cells appeared to be 10–30% of that detectable in cell lines derived from the gastrointestinal tract (such as T84 and CaCo-2) or in stably transfected CHO cells as estimated by Western-blot analysis (Figure 2). Interestingly, MDCK type II cells, which originate from the proximal tubule of the kidney [42], do not appear to express a measurable amount of CFTR immunoreactive protein, as assayed with M3A7, L12B4 (Figure 2) or 24-1 antibodies.

Expression of membrane proteins is known to depend on the degree of polarization of epithelia. To examine whether expression of CFTR protein can be detected in MDCK II cells under highly polarized conditions, MDCK cells were maintained at confluence on plastic or permeable filter supports for 1 week. Western-blot analysis with the M3A7 antibody showed that CFTR protein expression was elevated in MDCK I cells but remained undetectable in MDCK II cells (Figure 3). Meanwhile, MDCK I and II cells displayed similar levels of expression of the Na/K-ATPase α subunit (Figure 3).

MDCK I cells display a cAMP-stimulated iodide conductance with pharmacological characteristics similar to those of the CFTR

To assess whether CFTR expression confers cAMP-stimulated anion conductance, the plasma membrane iodide permeability was determined. Following the replacement of intracellular chloride by iodide, the rate of cAMP-stimulated iodide exit from the cytosol to the extracellular space was monitored [34]. Elevation of the cytosolic cAMP concentration with a combination of forskolin, dibutyryl-cAMP and IBMX substantially increased the rate of iodide release from MDCK I monolayers (Figure 4, left panel). Similar results were obtained with the

Figure 4 PKA activation stimulates iodide efflux from MDCK I cell monolayers

Iodide efflux assays were performed on confluent MDCK I cells (left panel) grown on a plastic support and on CHO cells expressing the CFTR (right panel), as described in the Materials and methods section. The amount of iodide released was quantified using an iodide-selective electrode. Activation of PKA was evoked by addition of an agonist cocktail (0.5 mM dibutyryl-cAMP, 0.2 mM IBMX and 20 μ M forskolin in DMSO) to the efflux medium at the 0 min time point (●). In the control cells, the cocktail was replaced by an equivalent amount of DMSO (○). Representative results of three independent experiments are shown. Data points are means of triplicate determinations with S.E.M.s of < 5%.

Table 1 Effect of PKA activation on iodide efflux from MDCK I and MDCK II cells

The rate of iodide efflux from MDCK I and MDCK II cells grown on a plastic or permeable support for 2 or 7 days post-confluence respectively was measured as described in the Materials and methods section. PKA was stimulated with forskolin (20 μ M) in MDCK I cells, while a combination of forskolin (20 μ M), IBMX (0.2 mM) and 8-(4-chlorophenylthio)-cAMP (0.5 mM) was applied to attain maximal stimulation in MDCK II cells. The iodide efflux rate represents the amount of iodide released during the second minute of PKA stimulation (means $\pm$ S.E.M.). For comparative purposes, iodide release from filter-grown MDCK cells was normalized to the area of the plastic support.

Cells	Culture support	Iodide efflux (nmol/min)		n
		Apical	Basolateral	
MDCK I	Plastic	17.8 $\pm$ 1.3	—	12
MDCK II	Plastic	0.28 $\pm$ 0.06	—	9
MDCK I	Filter	29.0 $\pm$ 2.2	1.0 $\pm$ 0.6	9
MDCK II	Filter	0.33 $\pm$ 0.20	0.0	3

Table 2 Effects of anion-channel inhibitors on cAMP-stimulated iodide efflux from MDCK I monolayers

MDCK I cells were cultured for 2 days after reaching confluence on a plastic support, and PKA-stimulated iodide efflux was determined as detailed in the Materials and methods section. The percentage inhibition was calculated by comparison with the iodide efflux value after 2 min of stimulation with 20 μ M forskolin (means $\pm$ S.E.M.). Inhibitors were included during the entire period of efflux measurements. The mean iodide release rate was 17.8 $\pm$ 1.3 nmol/min (n = 12).

Inhibitor	Concentration (μ M)	Inhibition (%)	n
Control	—	0 $\pm$ 6.0	12
NPPB	20	76.0 $\pm$ 5.0	3
	40	78.0 $\pm$ 4.7	3
	100	81.5 $\pm$ 2.6	3
	200	87.0 $\pm$ 1.2	3
Glybenclamide	200	30.0 $\pm$ 4.2	3
	500	77.0 $\pm$ 2.9	9
DPC	200	60.0 $\pm$ 5.0	3
DIDS	500	0.0 $\pm$ 12.0	6

application of forskolin alone (not shown). The rate of iodide efflux from MDCK I cells was lower than that recorded in T84 cells (results not shown), or in CHO cells heterologously expressing the CFTR (Figure 4, right panel). This difference in the rate of iodide efflux is in keeping with the significantly lower level of expression of the CFTR in MDCK I cells (Figure 2). Fully consistent with the results of the CFTR immunoblots, MDCK II cells did not display cAMP-stimulated anion conductance, suggesting that CFTR expression is differentially regulated along the kidney tubule (Table 1).

To reinforce the notion that the CFTR mediates the observed cAMP-stimulated anion conductance, the effects of anion-channel inhibitors were examined. Although there is no highly specific CFTR channel inhibitor available, DPC, NPPB and glybenclamide blocked our observed cAMP-stimulated iodide release at concentrations that have been used previously to inhibit CFTR channel activity (Table 2; see, e.g., [43–45]). Additionally, DIDS, a stilbene derivative, was found not to interfere with the cAMP-induced iodide efflux (Table 2). This inhibitor profile reflects the published inhibitor sensitivity of CFTR chloride channels [43–45].

The results of the immunoblot analyses, iodide efflux measure-

Figure 5 Functional CFTR is associated with the apical membranes of MDCK I cells

(A) Iodide efflux to the apical (●) and basolateral (○) compartments was measured as described in the Materials and methods section for MDCK I cells cultured on a permeable filter support. Stimulation of PKA was attained by the addition of 0.5 mM dibutyryl-cAMP, 0.2 mM IBMX and 20 μ M forskolin in efflux medium at 0 min. Data points are means of triplicate determinations. (B) Localization of the CFTR by domain-specific cell-surface biotinylation. The apical or basolateral plasma membrane of filter-grown MDCK I cells was covalently labelled with NHS-SS-biotin. Apical (lane 1) or basolateral (lane 2) biotinylated proteins were isolated on a monomeric avidin column, immunoprecipitated with the anti-CFTR antibodies M3A7 and L12B4 (1 μ g/ml each) and phosphorylated *in vitro* with [γ - 32 P]ATP in the presence of the catalytic subunit of PKA. Phosphorylated proteins were separated on an SDS/6%-PAGE gel. Lane 3 represents the phosphorylation profile of the whole-cell lysate.

ments and inhibitor sensitivity profiles together strongly suggest that the CFTR confers cAMP-regulated anion conductance to the plasma membrane of MDCK I cells. However, we were unable to observe CFTR expression and cAMP-stimulated chloride conductance in PANC-1 or RD2 monolayers, despite the presence of detectable amounts of CFTR transcripts (results not shown). This may be explained by the different detection limits of the RT-PCR and immunochemical assays.

Polarized distribution of the CFTR in MDCK I cells

Immunocytochemical and electrophysiological analyses of CFTR-expressing native epithelia, immortalized cell lines and transfected cells suggest that the CFTR is localized to the apical surface and is absent from the basolateral domain of secretory epithelia [11–13]. This polarized distribution of the CFTR seems to be essential for mediating chloride secretion [4]. To assess the subcellular localization of the CFTR in MDCK I cells, the following biochemical and functional studies were undertaken. MDCK cells were grown on a permeable filter support that allows independent access to the apical and basolateral surfaces. After the development of a tight monolayer, the anion conductance was assessed by measuring iodide release into the apical and basolateral compartments individually. Upon elevation of the intracellular cAMP concentration, iodide release into the apical but not the basolateral compartment was significantly stimulated (Figure 5A and Table 1). The lack of iodide release into the basolateral compartment could not be explained by a diffusional barrier, since nystatin permeabilization from the basolateral site evoked a significant amount of iodide release from MDCK I and II cells (results not shown). Taking into account that the electrochemical gradient for chloride ions is approximately the same across the basolateral and apical plasma

membranes, the results suggest that cAMP-regulated anion conductance resides in the apical membrane domain of MDCK I cells. In contrast, PKA activation failed to induce iodide efflux on filter-grown MDCK II cells (Table 1).

To verify the apical localization of the CFTR by biochemical means, we performed domain-selective biotinylation, a technique that is widely used to assess the asymmetrical distribution of membrane proteins in polarized epithelia. [31,32]. Confluent monolayers of filter-grown MDCK I cells were biotinylated with NHS-SS-biotin from either the apical or the basolateral compartment. Biotinylated proteins were then isolated on a monomeric avidin column [32]. Subsequently, the CFTR was immunoprecipitated from the effluent and phosphorylated *in vitro* with the catalytic subunit of PKA in the presence of [γ - 32 P]ATP. Phosphorylated CFTR could be recognized in those samples that contained biotinylated proteins from the apical surface, but not from the basolateral surface (Figure 5B). Since the apical and basolateral surfaces have similar accessibility to biotin derivatives (e.g. [46]), our results imply that functional CFTR resides predominantly in the apical plasma membrane of MDCK I cells, consistent with its reported subcellular localization in all secretory epithelia that have been investigated to date.

DISCUSSION

In order to establish a tissue culture model for investigating the role of the CFTR in kidney physiology, expression of the CFTR in various renal cell lines was examined. Several lines of evidence suggest that functional CFTRs are constitutively expressed at the apical membrane of the highly differentiated MDCK I cells. First, CFTR mRNA could be identified by RT-PCR utilizing two different primer sets. Secondly, using three different monoclonal antibodies raised against the CFTR, a polypeptide band with an apparent molecular mass of 170 kDa, and with heterogeneity similar to that of complex-glycosylated CFTR, could be visualized in whole-cell extracts. The slight difference in the apparent molecular mass of the CFTR in MDCK I cells could be due to a different extent of post-translational modification(s). Thirdly, domain-selective biotinylation in conjunction with immunoprecipitation and *in vitro* phosphorylation revealed that the CFTR resides predominantly in the apical plasma membrane of MDCK I cells grown on permeable supports. Accordingly, elevation of the cytosolic cAMP concentration increased the apical, but not the basolateral, anion conductance. Finally, the inhibitor profile of cAMP-stimulated iodide efflux was consistent with the operation of CFTR chloride channels. Interestingly, CFTR expression was not detectable in other kidney-derived cell lines of epithelial (LLC-PK) or non-epithelial (BHK, HEK-293) origin, suggesting that expression of the CFTR might be restricted to specific regions or cells of the kidney ([20,39], and results not shown). Similarly, we were unable to detect CFTR expression by either biochemical or functional assays in MDCK type II cells, which originate from the proximal tubule [42]. This observation is consistent with the notion that the apical and basolateral membranes of the proximal tubule show negligible chloride conductance, besides that induced by swelling and depolarization [49], and suggest that CFTR expression is differentially regulated along the nephron.

Why has the CFTR protein remained undetectable in MDCK I cells? One possible explanation is that the relatively low level of CFTR expression is close to the detection limit of immunochemical assay [11–13,19]. A similar phenomenon was observed in airway surface epithelia, where functional CFTR was identified, yet immunochemical detection of the protein was relatively difficult [12,19]. We cannot formally exclude the possibility that

CFTR expression is induced upon exposing MDCK I cells to various tissue culture conditions. However, this is probably not the case, given the results of electrophysiological experiments which indicate that low-conductance anion channels with CFTR-like characteristics are expressed in primary cultures of inner medullary collecting duct cells and in distal convoluted tubule cells [24–26]. Furthermore, the CFTR was functionally and immunologically localized to the cyst-lining epithelia in primary cell cultures from patients suffering from the autosomal dominant polycystic kidney disease [47]. In this latter study, the authors proposed that the CFTR contributes to cyst formation by mediating transepithelial chloride transport [47]. Our observation that apically located CFTR is responsible for cAMP-stimulated chloride secretion in MDCK I cells is consistent with these electrophysiological studies and with earlier findings that documented the role of intracellular cAMP in the fluid secretion of the MDCK cyst model [48].

Why is there no pathological consequence in the kidney of CF mutation if the CFTR plays a role in the chloride transport of the distal tubule? In view of the multitude of chloride channels that have been described in the distal tubule [49,50], one possible explanation is that the chloride transport activity of the CFTR represents only a fraction of the total chloride transport capacity. Alternatively, we can invoke a compensatory up-regulation of other chloride channel(s) similar to that observed for the Ca^{2+} -activated Cl^- conductance which becomes more active in the respiratory epithelia of CF patients and CF knock-out mice [51,52]. Further biochemical and electrophysiological studies are required to distinguish between these possibilities.

In conclusion, we propose that MDCK cells can serve as a useful tissue culture model in future studies to investigate the role and differential expression of the CFTR in the proximal and distal tubules under physiological and pathological conditions.

We thank Dr. Steen Hansen, Dr. Cindy Bell and Dr. Paul Quinton for kindly providing cell lines; Dr. Christine Bear and Dr. Michael Caplan for the 24-1 (anti-CFTR) and 6H (anti-Na/K-ATPase α subunit) antibodies respectively, Dr. Aleksander Edelman for the gift of NPPB and Agnes Ambrus for secretarial help. A.M. was supported by a CCFR Fellowship. S.G. is a Howard Hughes International Scholar, and G.L.L. was supported by a Scholarship from the Medical Research Council of Canada. This work was supported by MRC Canada, the Canadian Cystic Fibrosis Foundation and an N.I.H. grant. The instrumentation was funded in part by an Ontario Block Term Grant.

REFERENCES

- Rommens, J. M., Iannuzzi, M. C., Kerem, B.-S., Drumm, M. L., Melmer, G., Dean, M., Rozmahel, P., Cole, J. L., Kennedy, D., Hidaka, N. et al. (1989) *Science* **245**, 1059–1065.
- Riordan, J. R., Rommens, J. M., Kerem, B.-S., Alon, N., Rozmahel, R., Grzelczak, Z., Zielenski, J., Lok, S., Plavsic, N., Chou, J.-L. et al. (1989) *Science* **245**, 1066–1073.
- Boat, T. F., Welsh, M. J. and Beaudet, A. L. (1989) in *The Metabolic Basis of Inherited Disease*, 6th edn. (Scriver, C. R., Beaudet, A. L., Sly, W. S. and Valle, D., eds.), pp. 2649–2680, McGraw-Hill, New York.
- Quinton, P. M. (1990) *FASEB J.* **4**, 2709–2717.
- Tsui, L.-C. and Buchwald, M. (1991) *Adv. Hum. Genet.* **20**, 153–266.
- Welsh, M. J. and Smith, A. E. (1993) *Cell* **73**, 1251–1254.
- Riordan, J. R. (1993) *Annu. Rev. Physiol.* **55**, 609–630.
- Higgins, C. F. (1992) *Annu. Rev. Cell Biol.* **8**, 67–113.
- Cutting, G. R. (1993) *J. Bioenerg. Biomembr.* **25**, 7–10.
- Tsui, L.-C. (1995) *Am. J. Respir. Crit. Care Med.* **151**, S47–S53.
- Puchelle, E., Gaillard, D., Ploton, D., Hinnrasky, J., Fuchey, C., Bouterin, M.-C., Jacquot, J., Dreyer, D., Parirani, A. and Dalemans, W. (1992) *Am. J. Respir. Cell. Mol. Biol.* **7**, 485–491.
- Kartner, N., Augustinas, O., Jensen, T., Naismith, A. L. and Riordan, J. (1992) *Nature Genet.* **1**, 321–327.
- Engelhardt, J. F., Yankaskas, J. R., Ernst, S. A., Yang, Y., Marino, C. R., Boucher, R. C., Cohn, J. A. and Wilson, J. M. (1992) *Nature Genet.* **2**, 240–248.
- Sood, R., Bear, C., Auerbach, W., Reyes, E., Jensen, T., Kartner, N., Riordan, J. R. and Buchwald, M. (1992) *EMBO J.* **11**, 2487–2494.

- 15 Nagel, G., Hwang, T.-C., Nastiuk, K. L., Nairn, A. C. and Gadsby, D. (1992) *Nature* (London) **360**, 81–84
- 16 Trezise, A. E. O., Linder, C. C., Grieger, D., Thompson, E. W., Meunier, H., Griswold, M. D. and Buchwald, M. (1993) *Nature Genet.* **3**, 157–164
- 17 Zeitlin, P. L., Crawford, I., Lu, L., Woel, S., Cohen, M. E., Donowitz, M., Montrose, M. H., Hamosh, A., Cutting, G. R., Gruenert, D. et al. (1992) *Proc. Natl. Acad. Sci. U.S.A.* **89**, 344–347
- 18 Ward, C. L., Krouse, M. E., Gruenert, D. C., Kopito, R. R. and Wine, J. J. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 5277–5281
- 19 Engelhardt, J. F., Zepeda, M., Cohn, J. A., Yankaskas, J. R. and Wilson, J. M. (1994) *J. Clin. Invest.* **93**, 737–749
- 20 McDonald, T. V., Nghiem, P. T., Gardner, P. and Martens, C. L. (1992) *J. Biol. Chem.* **267**, 3242–3248
- 21 Kopelman, H., Gauthier, C. and Bornstein, M. (1993) *J. Clin. Invest.* **91**, 1253–1257
- 22 Crawford, I., Maloney, P. C., Zeitlin, P. L., Guggino, W. B., Hyde, S. C., Turley, H., Gatter, K. C., Harris, A. and Higgins, C. F. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 9262–9266
- 23 Tata, F., Stainer, P., Wicking, C., Halford, S., Kruyer, H., Lench, N. J., Scambler, P. J., Hansen, C., Braman, J. C., Williamson, R. and Wainwright, B. J. (1991) *Genomics* **10**, 301–307
- 24 Ling, B. N., Kokko, K. E. and Eaton, D. C. (1994) *J. Clin. Invest.* **93**, 829–837
- 25 Poncet, V., Tauc, M., Bidet, M. and Poujeol, P. (1994) *Am. J. Physiol.* **266**, F543–F553
- 26 Husted, R. F., Volk, K. A., Sigmund, R. D. and Stokes, J. B. (1995) *J. Clin. Invest.* **95**, 644–650
- 27 Lukacs, G. L., Chang, X.-B., Bear, C., Kartner, N., Mohamed, A., Riordan, J. R. and Grinstein, S. (1993) *J. Biol. Chem.* **268**, 21592–21598
- 28 Mohamed, A. and Jansson, C. (1989) *Plant Mol. Biol.* **13**, 693–700
- 29 Denning, G. M., Anderson, M. P., Amara, J. F., Marshall, J., Smith, A. E. and Welsh, M. J. (1992) *Nature* (London) **358**, 761–764
- 30 Lukacs, G. L., Mohamed, A., Kartner, N., Chang, X.-B., Riordan, J. R. and Grinstein, S. (1994) *EMBO J.* **13**, 6076–6086
- 31 Le Bivic, A., Real, F. X. and Rodriguez-Boulan, E. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 9313–9317
- 32 Prince, L. S., Workman, P. R. and Marchese, R. B. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 5192–5196
- 33 Cheng, S. H., Rich, D. P., Marshall, J., Gregory, R. J., Welsh, M. J. and Smith, A. E. (1991) *Cell* **66**, 1027–1036
- 34 Chang, X.-B., Tabcharani, J. A., Hou, Y.-X., Jensen, T. J., Kartner, N., Alon, N., Hanrahan, J. W. and Riordan, J. R. (1993) *J. Biol. Chem.* **268**, 11304–11311
- 35 Cozens, A. L., Yezzi, M. J., Kunzelmann, K., Ohri, T., Chin, L., Finkbeiner, W. E., Widdicombe, J. H. and Gruenert, D. C. (1994) *Am. J. Respir. Cell. Mol. Biol.* **10**, 38–47
- 36 Koh, J., Sierra, T. J. and Collins, F. S. (1993) *J. Biol. Chem.* **268**, 15912–15921
- 37 Trapnell, B. C., Chu, C.-S., Paako, P. K., Banks, T. C., Yoshimura, K., Ferrans, V. J., Chernick, M. S. and Crystal, R. G. (1991) *Proc. Natl. Acad. Sci. U.S.A.* **88**, 6565–6569
- 38 Ward, C. L. and Kopito, R. (1994) *J. Biol. Chem.* **269**, 25710–25718
- 39 Marshall, J., Fang, S., Ostedgaard, L. S., O'Riordan, C. R., Ferrara, D., Amara, J. F., Hoppe, H., Scheule, R. K., Welsh, M. J., Smith, A. E. and Cheng, S. H. (1994) *J. Biol. Chem.* **269**, 2987–2995
- 40 Trezise, A. E. O. and Buchwald, M. (1991) *Nature* (London) **353**, 434–437
- 41 Yoshimura, K., Chu, C.-S. and Crystal, R. G. (1993) *J. Biol. Chem.* **268**, 686–690
- 42 Richardson, J. C. W., Scalera, V. and Simmons, N. L. (1981) *Biochim. Biophys. Acta* **673**, 26–36
- 43 Gray, M. A., Pollard, C. E., Harris, A., Coleman, L., Greenwell, J. R. and Argent, B. E. (1990) *Am. J. Physiol.* **259**, C752–C761
- 44 Hasegawa, H., Skach, W., Baker, O., Calayag, M. C., Lingappa, V. and Verkman, A. S. (1992) *Science* **258**, 1477–1479
- 45 Sheppard, D. N. and Welsh, M. J. (1992) *J. Gen. Physiol.* **100**, 573–591
- 46 Matter, K., Whitney, J. A., Yamamoto, E. M. and Mellman, I. (1993) *Cell* **74**, 1053–1064
- 47 Hanaoka, K., Devuyst, O., Schweibert, E. M., Wilson, P. D. and Guggino, W. B. (1996) *Am. J. Physiol.* **270**, C389–C399
- 48 Mangoo-Karim, R., Uchic, M., Lechene, C. and Grantham, J. J. (1989) *Proc. Natl. Acad. Sci. U.S.A.* **86**, 6007–6011
- 49 Palmer, L. G. (1992) *Annu. Rev. Physiol.* **54**, 51–66
- 50 Jentsch, T. J. (1995) *J. Membr. Biol.* **144**, 189–197
- 51 Johnson, L. G., Boyles, S. E., Wilson, J. and Boucher, R. (1995) *J. Clin. Invest.* **95**, 1377–1387
- 52 Clark, L. L., Grubb, B. R., Yankaskas, J. R., Cotton, C. U., McKenzie, A. and Boucher, R. C. (1994) *Proc. Natl. Acad. Sci. U.S.A.* **91**, 479–483
- 53 Gottardi, C. J. and Caplan, M. J. (1993) *Science* **260**, 552–554