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Shared apical sorting of anion exchanger isoforms AE2a, AE2b$_1$, and AE2b$_2$ in primary hepatocytes

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Abstract

AE2 (SLC4A2) is the member of the Na^+-independent anion exchanger (AE) family putatively involved in the secretion of bicarbonate to bile. In humans, three variants of AE2 mRNA have been described: the full-length transcript AE2a (expressed from the upstream promoter in most tissues), and alternative transcripts AE2b$_1$ and AE2b$_2$ (driven from alternate promoter sequences in a tissue-restricted manner, mainly in liver and kidney). These transcripts would result in AE protein isoforms with short N-terminal differences. To ascertain their translation, functionality, and membrane sorting, we constructed expression vectors encoding each AE2 isoform fused to GFP at the C-terminus. Transfected HEK293 cells showed expression of functional GFP-tagged AE2 proteins, all three isoforms displaying comparable AE activities. Primary rat hepatocytes transfected with expression vectors and repolarized in a collagen-sandwich configuration showed a microtubule-dependent apical sorting of each AE2 isoform. This shared apical sorting is liver-cell specific, as sorting of AE2 isoforms was basolateral in control experiments on polarized kidney MDCK cells. Hepatocytic apical targeting of AE2 isoforms suggests that they all may participate in the canalicular secretion of bicarbonate to bile.

Keywords: Alternate promoters; Biliary bicarbonate secretion; HEK293 cells; Kidney epithelial MDCK cells; Sandwiched repolarized hepatocytes

Na^+-independent anion exchangers (AE) are multispanning membrane proteins that mediate electroneutral and reversible exchange of Cl^- and HCO_3^- across the plasma membrane, the direction of net transport being usually Cl^- influx and HCO_3^- efflux [1,2]. In cooperation with other ion carriers, AE proteins are involved in intracellular pH and cell volume regulation, as well as in transepithelial acid–base transport [2]. Thus, in hepatobiliary cells, AE carriers have been postulated as important regulators of bicarbonate content in bile [3].

To date, four members of the AE family (AE1, AE2, AE3, and AE4) have been characterized in several species [1,4–6]. Although AE genes locate in different chromosomes they all have a similar structure. A frequent feature for AE genes is the usage of alternate promoters that leads to the expression of transcript variants, often in a tissue- and cell-specific manner [1,7–9]. With respect to AE2, three transcript variants have been reported in humans: the full-length message AE2a and the alternative isoforms AE2b$_1$ and AE2b$_2$ [9]. AE2a mRNA is transcribed from the upstream promoter in most human tissues (liver, stomach, thyroid, prostate, and kidney), while the two alternative isoforms are expressed in a more tissue-restricted fashion (mainly in liver and kidney), from AE2b$_2$/AE2b$_1$ overlapping promoter sequences within intron 2 of the AE2 gene [9]. Alternative transcriptions result in different first exons leading to short 5′-diversities in the respective open reading frames [9]. Thus, the first 17 amino acid triplets of AE2a are replaced by 3 triplets for residues MTQ in AE2b$_1$ and by 8 triplets for residues MDFLLRPQ in AE2b$_2$. In addition to the mRNA level [9–11], liver

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expression of the human AE2 gene has been substantiated at the protein level by using a monoclonal antibody against an AE2 peptide common to all three AE2 isoforms [12]. This study showed AE2 immunoreactivity restricted to the lumenal surface of the hepatobiliary tree [12]. Such an apical location of AE2 in liver cells is compatible with the reported apical AE activity seemingly involved in the biliary bicarbonate secretion [3,13–16]. Although in baseline conditions, expression of alternative transcripts $AE2b_1$ and $AE2b_2$ each accounts for about 10% of the full-length AE2a message in the human liver [9], our recent finding that HNF1α may transactivate alternative expression [17] suggests that the physiological significance of alternative AE2 messages might be related to the possibility for liver cells to increase their expression when needed for bicarbonate secretion. But whether $AE2b_1$ and $AE2b_2$ transcripts are indeed translated into proteins and display AE transport activity in mammalian cells and whether they are apically sorted in liver cells needed to be investigated. Thus far, the progress in these aspects has been hampered by the difficulty to obtain antibodies specific for each AE2 isoform due to their limited amino acidic differences. Here, we have set up cultured cell models and used recombinant GFP-tagged AE2 isoforms to address these issues. We found that AE2 isoforms may all be expressed in HEK293 cells, in which they are able to perform Cl^-/HCO_3^- exchange at similar rates. Transfection and further repolarization of primary cultured rat hepatocytes indicated that each AE2 isoform is sorted to the apical pole of hepatocytes. Such an apical sorting is liver-cell specific as the same isoforms are targeted to the basolateral domain in polarized kidney MDCK cells. Thus, our present data support the view that all three AE2 isoforms may contribute to bile formation by mediating HCO_3^- secretion at the canalicular membrane of hepatocytes.

Materials and methods

Culture media, fetal bovine serum (FBS), and antibiotics/antimycotic were from Gibco, while chemicals and hormones were purchased from Sigma (except otherwise stated). All used animals were kept under conventional housing conditions and received humane care according to institutional guidelines.

Production of expression vectors for AE2–GFP fusion proteins. DNA amplicons relative to each human AE2 isoform cDNA were produced by RT-PCR on total RNA from HepG2 cells. Also, DNA fragments including short regions of isoform-specific promoters and corresponding initial exons were obtained by PCR on cosmid HAE2co5 with the human AE2 gene [18]. To obtain the three AE2 isoform cDNAs with their related proximal promoter regions, overlapping amplicons were used as template for further megaprimer reactions [19], with flanking oligonucleotides (and the *Pfu* DNA polymerase). The downstream primer 5′-AGTACAATGAGATGCCC ATGCCTGTGCTCGAG was common for all three N-terminal variants and included an *Xho*I restriction site (underlined) replacing the stop codon. Directional subcloning of isoform-specific promoter/cDNA fragments into *Eco*47III/*Xho*I digested pEGFP-N1 vector (Clontech) was achieved by using particular restriction sites in the proximal promoter regions (*Sac*I in AE2a, *Ssp*I in $AE2b_1$, and *Bsu*36I in $AE2b_2$, followed by blunt-end production with mung bean nuclease) and the downstream *Xho*I site. This resulted in recombinant plasmids for the expression of AE2 isoforms fused—through a linker of 22 amino acids—to GFP at their C-termini, under control of CMV early promoter and enhancer. The integrity of inserts was ascertained by sequence analysis. After their extraction with Nucleobond AX500 kit (Clontech) plasmids were further purified by phenol-extraction/ethanol-precipitation and resuspended in TE buffer.

Stable transfection of HEK293 cells. Cells (1.25×10^5) were seeded in 35-mm wells, incubated for 48 h, and transfected with 1 μg of the respective AE2–GFP construct (previously linearized with *Apa*LI) and 1.2 μg of carrier DNA by using the Calcium Phosphate Transfection Kit (Gibco). After 10–15 days in culture medium (DMEM with 10% FBS and 1× penicillin–streptomycin–amphotericin B) supplemented with 2 mg/mL G418 (Gibco), stably transfected clones were selected. Expression of AE2–GFP fusion proteins was estimated by flow cytometry using a FACSCalibur apparatus (BD Biosciences).

Measurement of AE activity. HEK293 cells (1.25×10^5) from stably transfected clones were seeded onto poly-L-lysine coated 12-mm coverslips 3–4 days before AE assays. AE activity was measured by microfluorimetric detection of pH_i changes as described [16]. Loading of cells with BCECF (Molecular Probes), calibration and maneuver procedures, and exchange calculation were all as reported for the Cl^--free KRB perfusion system [20]. Briefly, cells perfused with KRB were switched to a Cl^--free KRB, and the rate of intracellular alkalinization after extracellular Cl^- removal (owing to HCO_3^- influx in exchange with intracellular Cl^- outflow) was determined. These experiments were carried out both in the absence and in the presence of 200 μM DIDS.

Western blot analysis. HEK293 clones of stable transfectants showing high levels of GFP-tagged AE2 isoforms were cultured on 150×20 mm plates to 70–80% confluence. Collected cells were centrifuged for 5 min at 4 °C, and pellets were frozen in liquid nitrogen and stored at −80 °C. To obtain a positive control for immunodetection of GFP alone, HEK293 cells were transiently transfected with the empty CMV-GFP vector by the calcium-phosphate protocol, being in culture for 48 h until their freezing and storage. Protein extracts were prepared for all samples by adding 1 mL of denaturing buffer (10 mM Tris, pH 7.5, 8 M urea, and 1% SDS) to frozen cells, followed by sonication. After 30-min centrifugation (13,000 rpm at 4 °C), supernatants were collected, aliquoted, and preserved at −80 °C. Protein extracts were run in 0.1% SDS–7.5% PAGE and electrotransferred to a nitrocellulose membrane. After blocking in 5% nonfat dried milk powder in PBS with 0.5% Tween, membranes were incubated with mouse monoclonal antibody IgG1 against GFP (Molecular Probes) diluted 1:1000, followed by incubation with peroxidase-conjugated secondary goat anti-mouse antibody (Pierce) diluted 1/5000, and further visualization of bands with a Perkin–Elmer Life Sciences Kit.

Transient transfection and repolarization of cultured primary rat hepatocytes. Hepatocytes were isolated from male Wistar rats (200–300 g bw) by perfusion with Collagenase-Hepatocyte Qualified (Gibco). Cells with a viability >85% (determined by trypan blue exclusion) were plated onto Permanox-chamber slides (Nunc), in a collagen-sandwich configuration as described [21], with a few modifications to allow for their transfection. Briefly, slides were precoated with gelled collagen (800 μL of rat tail collagen type I from BD Biosciences mixed with 100 μL of 0.1 M NaOH and 100 μL of 10× DMEM), gelatinized for 1 h at 37 °C. Hepatocytes were seeded on precoated slides (1.5×10^6 cells/well) and incubated for 2 h at 37 °C in RPMI 1640 medium with FBS (10%) and antibiotics. Afterwards, the medium was replaced with fresh warm RPMI 1640 medium with FBS (2%), antibiotics, dexamethasone 400 μg/mL, and insulin 4 μg/mL. We optimized transfection of primary hepatocytes by trying six different commercial reagents under multiple conditions, and found the highest efficiency

(5–10%) with Tfx-50 Reagent (Promega) employed in a 4:2 ratio to DNA. Thus, we used 10 μg of the reagent with 5 μg DNA (0.3 μg of specific AE2 expression plasmid and 4.7 μg of carrier DNA) per well. DNA and reagent were diluted in 75 μL RPMI 1640 medium and incubated for 10 min to allow complexes to form. Hepatocytes cultured on gelled-collagen for 24 h were washed and 750 μL of RPMI 1640 medium was added followed by dropwise addition of the transfection mixture, incubated for 2 h, and 750 μL of RPMI 1640 medium with FBS (4%), 2× antibiotics, insulin 8 μg/mL, and dexamethasone 800 μg/mL was added. One day after transfection, hepatocytes were washed and overlaid with collagen gel, the formation of biliary canaliculi being monitored by phase-contrast microscopy. Four days after transfection (i.e., 3 days after collagen overlay or 5 days after plating), morphological polarization was ascertained by immunodetection of the canalicular marker dipeptidylpeptidase IV (DPPIV; the antibody was kindly provided by Dr. A. Quaroni, Cornell University, Ithaca, NY). For routine canalicular visualization, staining of actin microfilaments with phalloidin–TRITC labeled (Sigma) was carried out. Cells were fixed with 3.7% paraformaldehyde in PBS, permeabilized with 0.1% Triton X-100 in PBS, and incubated 30 min with labeled phalloidin (100 ng/mL). After detaching chambers and mounting slides with Vectashield (Vector Laboratories), fluorescent signals were observed under an epifluorescence microscope. For microtubule-disruption studies, repolarized hepatocytes were treated for 4 h before fixation with 10 μM colchicine (Sigma) in its current culture medium.

Transient transfection and repolarization of MDCK cells. MDCK cells were seeded onto Permanox-chamber slides at 5×10^5 cells/well and cultured in Earle's MEM with FBS (10%) and antibiotics. After 24 h, 90–95% confluent cells were transfected with expression vectors by using Lipofectamine 2000 Reagent (Gibco). The transfection mixture (a final volume of 750 μL/well of Opti-MEM with 9.45 μL of the transfection reagent, 0.17 μg of the respective AE2–GFP construct, and 1.51 μg of carrier DNA) was added dropwise to cells and left to stand for 4 h, when it was replaced by culture medium. MDCK cells were grown to form a continuous monolayer of polarized cells. Polarization was assessed by immunodetection of the apical marker gp114 and the basolateral marker gp58 (with antibodies kindly provided by Dr. I. Mellman, Yale University, New Haven, CT), through differential antigen accessibility with and without permeabilization of plasma membranes (4-min incubation at room temperature in PBS with 0.1% Triton X-100). Fluorescent signals in transfected and polarized cells were visualized under an epifluorescence microscope.

Results and discussion

Functional expression of AE2 isoforms in HEK293 cells

cDNAs for human N-terminal variants AE2a, $AE2b_1$, and $AE2b_2$ were subcloned into pEGFP-N1 vector in order to obtain CMV-driven expression of C-terminal-tagged AE2 isoform proteins in mammalian cells. To evaluate the functional expression of these isoforms we used non-polarized HEK293 cells which had been consistently employed to test AE carriers [22–25], as they show no endogenous AE activity and have almost negligible intrinsic buffering capacity [22]. After producing stable transfectants of HEK293 cells for each AE2 isoform, flow cytometry showed a wide variation in the intensity of fluorescent signals between clones. Variable expression was not related to the type of AE2 isoform–GFP fusion protein, and was most probably due to a random integration of recombinant DNA in the HEK293 genome. Immunoblot detection of recombinant fusion proteins with an antibody against GFP revealed bands of ~200 kDa (Fig. 1A), that is the

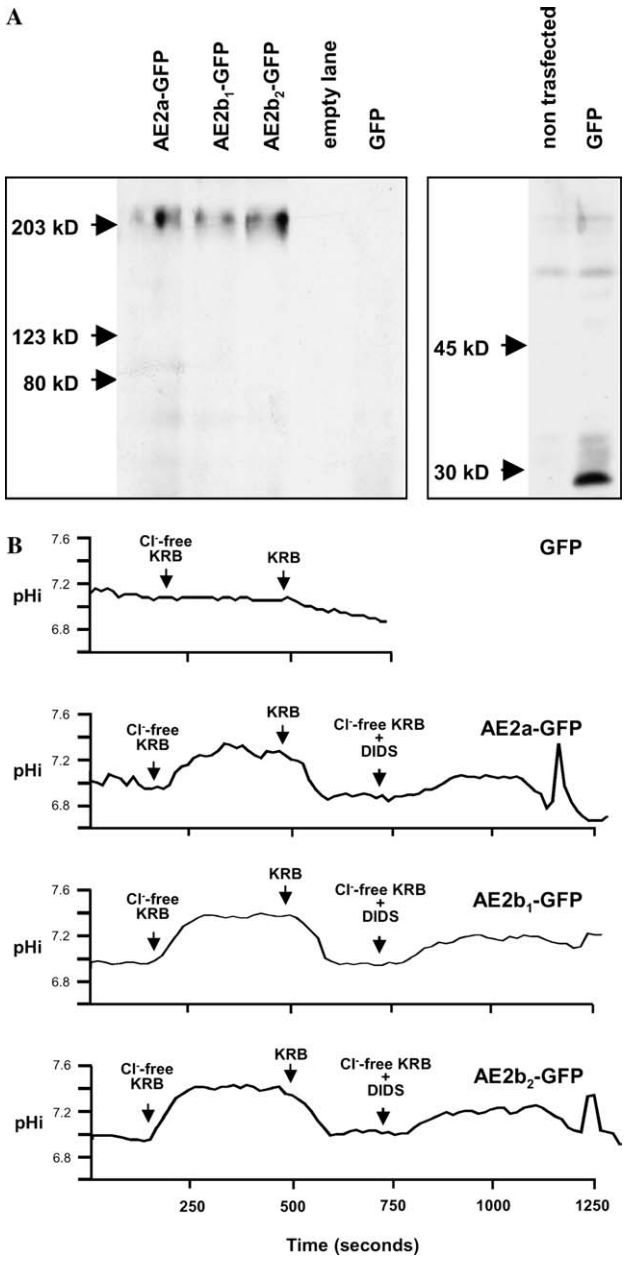

Fig. 1. Protein expression and functionality of GFP-tagged AE2 isoforms. (A) Immunoblot detection of AE2–GFP fusion proteins in protein extracts from stable transfectants of HEK293 cells by using an anti-GFP antibody. A total of 125 μg of protein extract for each sample was run in SDS–PAGE and electrotransferred onto a nitrocellulose membrane. The positive control to the right (GFP lanes) corresponds to extracts from HEK293 cells transiently transfected with the empty vector pEGFP-N1 expressing only GFP under the CMV promoter. (B) Typical profiles of AE activity in HEK293 cells stably transfected for each GFP-tagged AE2 isoform. Cells were first perfused with KRB and then switched to Cl^--free KRB as described [20]. These maneuvers were repeated in the presence of 200 μM DIDS. A negative control was carried out in HEK293 cells transfected with the empty vector pEGFP-N1 expressing only GFP under the CMV promoter.

expected size for each glycosylated AE2 isoform (~170 kDa) fused to GFP (27 kDa) through a 22-residue linker (2.5 kDa). These findings indicate that not only AE2a transcript but also alternative $AE2b_1$ and $AE2b_2$ variants are able to be translated into true protein isoforms in mammalian cells.

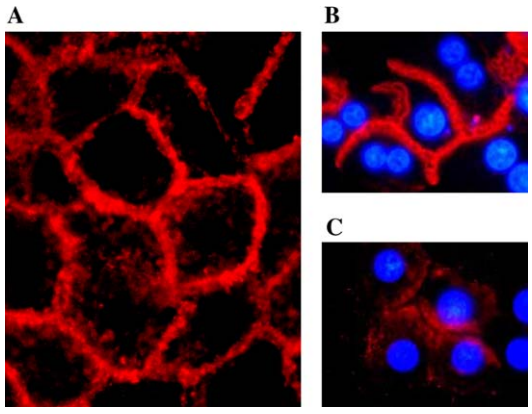

Fig. 2. Repolarization of primary rat hepatocytes after their transfection with Tfx50 Reagent and collagen-sandwich culture. (A) DPPIV immunofluorescence at the canalicular network of repolarized hepatocytes after 72 h in sandwich culture. (B) Canalicular network of hepatocytes sandwiched as in (A), after staining of the apical cytoskeleton with TRITC–phalloidin conjugate. (C) Hepatocytes sandwiched as in (A), showing immunohistochemistry for Rab11a, a marker at vesicles of the subapical compartment. Nuclei in (B,C) are stained with DAPI for their visualization.

AE activities were measured by microfluorimetry [20] in selected clones of stable transfectants expressing high levels of GFP-tagged AE2a, $AE2b_1$, and $AE2b_2$. In contrast to non-transfected HEK293 cells or to cells transfected with the GFP-expressing empty vector pEGFP-N1, clones expressing each AE2 isoform showed a similar profile of intracellular alkalinization upon maneuver procedures, consistent with a DIDS-inhibitable Na^+-independent Cl^-/HCO_3^- exchange (Fig. 1B). Repeated experiments on isoform-specific transfectants with equivalent expression levels (two clones for each AE2 isoform) resulted in a comparable basal AE activity. Moreover, HEK293 cells transiently transfected either with the same AE2-GPP plasmids employed for stable transfections or with plasmids for the expression of just each AE2 isoform without the GFP tag were determined for their AE activity, and no major differences were found between AE2 isoforms (not shown). Altogether, our data indicate that the three recombinant human AE2 isoforms display comparable AE activity regardless of the presence of the GFP tag, and suggest their correct folding and processing in mammalian cells such as the HEK293 cells.

Apical sorting of the three recombinant AE2 isoforms in polarized primary rat hepatocytes

To analyze the sorting of each AE2 variant in polarized liver cells we used the system of collagen-sandwiched

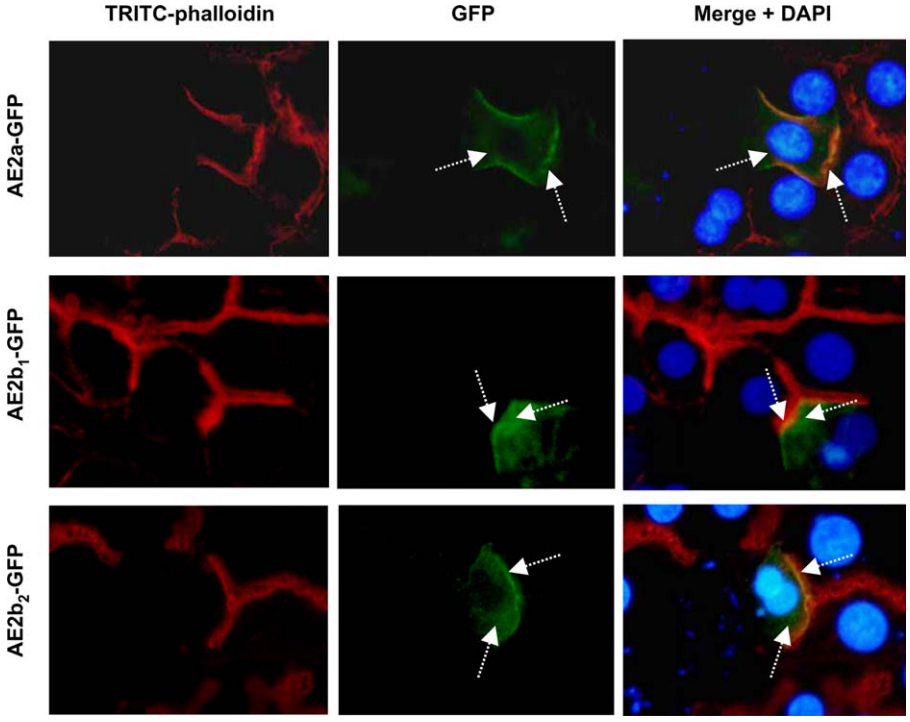

Fig. 3. Sorting of GFP-tagged AE2 isoforms in transfected primary rat hepatocytes repolarized after 72 h in collagen-sandwich culture. Left panels, TRITC–phalloidin staining of the canalicular network; middle panels, GFP fluorescence; right panels, merge in which nuclei stained with DAPI are also visualized. Both apical and subapical green fluorescence are denoted by arrows.

primary rat hepatocytes [21,26]. This has been considered an appropriate model to investigate the characteristics of differentiated hepatocytes [27], and their dynamics of repolarization [28]. We introduced some methodological modifications, setting up a feasible protocol for the transfection of primary rat hepatocytes prior to their repolarization, which further strengthened the capabilities of the sandwich model. One day after transfection hepatocytes were overlaid with gelled collagen and the repolarization process was monitored by immunofluorescent detection of polarity markers. Canalicular immunoreactivity for DPPIV (Fig. 2) showed a complete morphological repolarization by 72 h after collagen overlay (i.e., 96 h after transfection). At this time Rab11a, which is a marker related to the vesicular trafficking system between endosomal subapical compartments and the plasma membrane in hepatic cells [29], could be immunolocalized in subapical vesicle-like structures in sandwiched hepatocytes (Fig. 2), indicating that the sorting apparatus was completely polarized as well. For further experiments, polarized apical cytoskeleton was routinely detected through staining of apical actin microfilaments with TRITC-conjugated phalloidin, which allowed for consistent identification of the canalicular network in fully repolarized cultures (Fig. 2).

After 72–96 h in collagen-sandwich culture, the GFP signals from each recombinant AE2 isoform were found to colocalize with the apical TRITC signals from the phalloidin-stained canalicular cytoskeleton as well as in subapical vesicle structures, while no signals were detected at the basolateral pole (Fig. 3). The distribution of GFP signals in the subapical vesicular compartment, and former reports on a detrimental effect of colchicine on bicarbonate-stimulated AE activity in rat hepatocytes [3], suggested that targeting of AE2 isoforms may involve microtubule-associated vesicular transport and exocytotic insertion in the canalicular membrane. This view is also supported by recent evidence in cholangiocytes for the presence of AE2 in subapical vesicles (together with two functionally related proteins, i.e., the chloride channel CFTR and the water channel AQP1), that move in a microtubule-dependent manner to the apical membrane in response to secretory agonists [30]. Thus, in accordance with a participation of microtubules in AE2 distribution, we found that treatment with colchicine partially dispersed the fluorescent signals from GFP-tagged AE2 isoforms, and resulted in an extensive punctate fluorescence across the cytoplasm, the green fluorescence being often detected at the basolateral membrane as well (Fig. 4). This effect of colchicine on AE2 isoforms resembles what has been reported for transcytotic canalicular proteins such as the cell adhesion molecule cCAM105 [31].

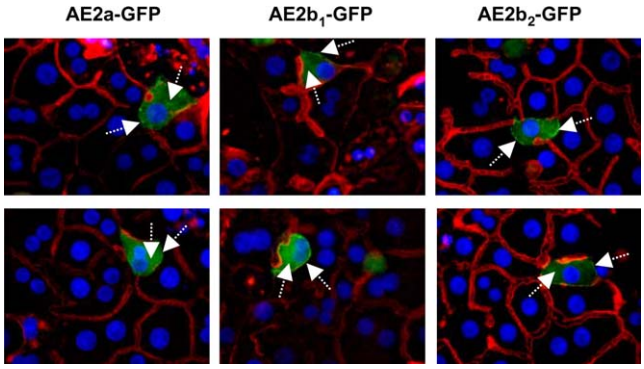

Fig. 4. Microtubule-disruption by colchicine. Primary rat hepatocytes transfected with AE2–GFP expression constructs and repolarized in collagen-sandwich culture for 72 h were further incubated with colchicine for 4 h. Nuclei stained with DAPI are visualized. A redistribution of GFP signals to the cytoplasm and to the basolateral membrane (indicated by arrows) can be observed.

Basolateral sorting of the three recombinant AE2 isoforms in polarized MDCK cells

As human alternative AE2 transcripts are expressed not only in the liver but also in the kidney [9], we used renal epithelial MDCK cells for control studies on cell specificity of polarized sorting of AE2 isofoms. MDCK cells seeded in near confluence were transiently transfected with each AE2–GFP expression vector and allowed to grow and form a monolayer of polarized cells. As expected for a correct repolarization, the apical marker gp114 was detected in the upper membrane domain and excluded from the lateral and basal surfaces under both permeabilizing and non-permeabilizing conditions, while the basolateral gp58 was only detected in the lateral and basal membranes under permeabilizing conditions (not shown). For each GFP-tagged AE2 isoform, fluorescent signals in these transfected kidney cells were always distributed along lateral and basal membranes (Fig. 5), coincident with the basolateral gp58 staining. Thus, all three AE2 isoforms share their sorting information to the basolateral domain in this non-hepatic control model of MDCK polarized cells. Such shared basolateral sorting of AE2–GFP isoforms

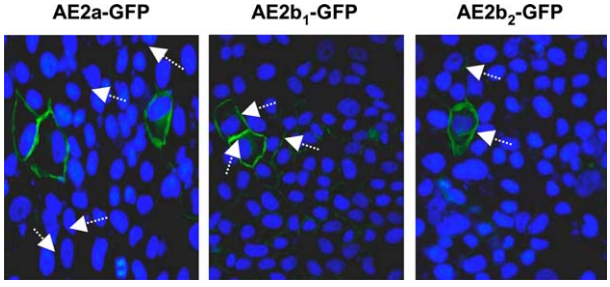

Fig. 5. Basolateral green fluorescence in polarized MDCK cells transiently transfected for each GFP-tagged AE2 isoform used as control. Nuclei are stained with DAPI for their visualization. Arrows indicate GFP signals at the basolateral domain.

in this kidney cell line is consistent with the reported basolateral localization of endogenous AE2 through immunohistochemistry on human kidney specimens by using an antibody common to the three isoforms [32].

Possible role of N-terminal variants of AE2 in hepatocytes

The occurrence of AE2 in the liver [11,12] strongly suggests that AE2 is the AE carrier putatively involved in the secretion of bicarbonate to bile. The fact that the three N-terminal variants AE2a, AE2b$_1$, and AE2b$_2$ are expressed in this tissue and that they all are targeted to the apical domain of hepatocytes may sound redundant. However, the physiological significance for this apparent redundancy might be related to differential regulation of the gene expression. While the AE2a upstream promoter seems to function in a constitutive fashion, overlapping alternate promoter sequences type "b" (within intron 2 of the AE2 gene) could be more responsive to regulatory mechanisms in hepatocytes. Our recent data on HNF1α being able to transactivate alternate expression [17] strengthen the view that alternative AE2 isoforms might be related to the possibility of liver cells increasing their expression in response to certain stimuli for bicarbonate secretion. Noticeably, the HNF1 consensus sequence found in the human alternate overlapping promoter within intron 2 is also observed in rat [7] and mouse [33] orthologs. Moreover, both alternative messages AE2b$_1$ and AE2b$_2$ are expressed in the liver in these three species, the rat included. Thus, using RT-PCR we could not only confirm the rat liver expression of AE2b$_1$ mRNA (previously referred to as just AE2b mRNA [7]) but also ascertain the liver expression of AE2b$_2$ mRNA (not shown).

Finally, our present data provide a clear evidence for the translation of the three N-terminal variant messages AE2a, AE2b$_1$, and AE2b$_2$ into similarly functional AE2 protein isoforms. The parallel and cell-type-specific sorting of these isoforms (all three isoforms are apical in hepatocytes and basolateral in kidney cells) suggests that no sorting signaling motifs are associated with the short isoform-specific N-terminal regions. Recent experiments in fibroblast-like cells suggest a role for the *trans*-Golgi-associated skeletal protein Ank$_{195}$ in the cell-type-specific sorting of AE2 to the Golgi in these non-polarized cells [34]. Which particular proteins may be involved in the cell-specific targeting of AE2 in polarized liver and renal cells deserves further investigation.

Acknowledgments

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