Targeting of the AE2 anion exchanger to the Golgi apparatus is cell type-dependent and correlates with the expression of Ank_{195}, a Golgi membrane skeletal protein

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Abstract Sodium-independent anion exchangers (AE1–4) show remarkable variability in their tissue-specific expression and subcellular localization. Currently, isoform-specific targeting mechanisms are considered to be responsible for this variable localization. Here, we report that targeting can also be cell type-specific. We show that the full-length AE2 protein and its green fluorescent protein- or DsRed-tagged variants localize predominantly either to the Golgi apparatus in COS-7 cells, or to the plasma membrane in HeLa cells. This alternative targeting did not seem to result from either translational or post-translational differences, but rather from differential expression of at least one of the Golgi membrane skeletal proteins, ankyrin$_{195}$ (Ank_{195}), between the two cell types. Comparative studies with several different cell lines revealed that the Golgi localization of the AE2 protein correlated strictly with the expression of Ank_{195} in the cells. The two Golgi-associated proteins also co-localized well and similarly resisted detergent extraction in the cold, whereas the plasma membrane-localized AE2 in Ank_{195}-deficient cells was mostly detergent-soluble. Collectively, our results suggest that Ank_{195} expression is a key determinant for the variable and cell type-dependent localization of the AE2 protein in the Golgi apparatus in mammalian cells.

Key words: Golgi apparatus; Protein targeting; Membrane trafficking; Ion transport; Cytoskeleton

1. Introduction

Na^+-independent anion exchangers (AE) are ubiquitously expressed transmembrane proteins that regulate intracellular pH, cell volume, and chloride concentration in cells by mediating an electroneutral exchange of chloride for bicarbonate. Currently, four distinct members (AE1–4) in this gene family have been identified and characterized [1–7]. Structurally, all AE proteins are type III membrane proteins with a large cytosolic N-terminal domain, and a C-terminal anion transport domain that consists of 12–14 membrane-spanning domains. The extreme C-terminus (~40 amino acids) is also cytosolic.

In most tissues, each gene gives rise into multiple N-terminally truncated variants either by means of tissue-specific mRNA splicing or by the use of alternative promoters [8,9]. The physiological role of these N-terminal truncations has remained obscure, but they may either have a regulatory role or they may possess specific targeting information needed for delivery of each of these variants to their correct destinations [8–10]. There is substantial evidence to support the latter possibility. For example, the N-terminally truncated mammalian AE1 and AE2 variants have been localized to the basolateral plasma membrane of kidney tubule epithelial cells [11], whereas the full-length AE2 (AE2a) isoform has been shown to localize to the apical plasma membrane in biliary and intestinal epithelial cells and in hepatocytes [12–14]. Second, of the four chicken erythrocyte N-terminal AE1 variants characterized thus far [10], only one with an additional exon is targeted to intracellular membrane systems, whereas the others are localized to the plasma membrane. Third, in many instances AE proteins are known to co-localize and to interact with the well-known cytoskeletal adapter proteins termed ankyrins [15–18]. This interaction is thought to be crucial for anchoring the AE1 and AE2 isoforms to the basolateral membrane of kidney β-intercalated cells and in gastric parietal cells. However, contradictory results for the AE2 protein have been reported in choroid plexus epithelial cells [19]. Fourth, the ankyrin binding sites in these proteins have been mapped within their variable N-terminal domains [20–23]. Because of this, the major ankyrin binding site in the N-terminally truncated kidney AE1 isoform is lost [24]. The kidney AE1, however, has been shown to bind to kanadaptin, a novel cytoplasmic protein expressed mainly in the kidney, liver, lungs, brain, and skeletal and cardiac muscle [25]. This interaction seems to be involved in localization of the AE1 in the apical, but not the basolateral, plasma membrane in kidney epithelial cells [25].

We [26] and others [10,11] have previously shown that some AE isoforms are also found in the membranes of the Golgi apparatus. Recently, we identified this Golgi-associated AE protein as the full-length AE2 (AE2a) isoform [27]. This finding was somewhat surprising, as it opposes the characteristic plasma membrane localization of this AE isoform in epithelial cells [11–14], including mammalian sperm cells, according to our own results [28,29]. However, there are two possibilities that could explain this apparent discrepancy in targeting of the same isoform in two different subcellular locations. One of these is that targeting is plastic and under control of external cues, as previously suggested [30]. The other possibility is that targeting is cell type-dependent. Given the co-localization and suggested cytoskeletal interactions in targeting of the plasma

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membrane-associated AE isoforms, and the known existence of two ankyrin isoforms [31,32] and one spectrin isoform [33,34] in the Golgi membranes, either possibility could involve reversible and/or regulated interactions with these Golgi membrane skeletal proteins.

In this report, we have addressed these issues by comparing the synthesis, processing, detergent resistance and targeting of the full-length AE2 protein and its green fluorescent protein (GFP)/DsRed-tagged variants in different cell types. Our results show that the AE2 isoform is indeed targeted in a cell type-dependent manner either to the Golgi apparatus or to the plasma membrane. The results also suggest that this differential targeting between the cell types depends on their differential expression of the trans-Golgi-associated ankyrin$_{195}$ (Ank$_{195}$) isoform.

2. Materials and methods

2.1. Antibodies and reagents

The antibody against the AE2 C-terminal peptide (amino acids 1229–1241) was prepared as previously described [28]. The pDs-Red-C1 vector, pE-GFP-C1 vector and the GFP antibodies were from Clontech. Alexa Fluor-conjugated secondary antibodies were from Molecular Probes (Eugene, OR, USA). The peroxidase-conjugated secondary antibodies were from P.A.R.I.S. (Compiègne, France). The antibodies against Golgi ankyrins, AnkG119 and Ank$_{195}$, were kind gifts from Drs. Jon Morrow (Yale University School of Medicine, New Haven, CT, USA) and Kenneth Beck (University of California, Davis, CA, USA).

2.2. Cell cultures and indirect immunofluorescence

COS-7 cells were grown in Dulbecco's modified Eagle's medium (DMEM) with Glutamax (Gibco, Grand Island, NY, USA) supplemented with 10% fetal calf serum and penicillin–streptomycin. HeLa cells were cultured in MEM with Glutamax supplemented with 10% fetal calf serum, penicillin–streptomycin and non-essential amino acids. CHO-K1, NRK, MDCK and MDBK cells were cultured in media recommended by ATCC.

Cells were processed for indirect immunofluorescence as described elsewhere [26]. Briefly, cells cultured on glass coverslips were washed two or three times with phosphate-buffered saline (PBS), and then fixed with 3.7% formaldehyde in PBS for 20–30 min. After washing with PBS, cells were immunostained with the antibodies in the presence of 0.1% saponin. Stained specimens were examined using an epifluorescence microscope and a CCD camera (Olympus BX-60 microscope).

2.3. GFP- and DsRed-AE2 fusion protein constructs and cell transfections

The GFP-AE2 fusion protein was prepared as described previously [28]. By using the same *Hin*dIII and *Eco*RI restriction enzymes, the full-length AE2a cDNA was also subcloned into the pDs-Red-C1 vector. Transfections with the plasmid constructs, pDs-Red-AE2 and pE-GFP-AE2 constructs (2 μg/plate), were done using the Fugene-6 transfection reagent (Roche Diagnostics) on cells plated 1 day earlier. Transfected cells were examined 24–48 h after the transfections.

2.4. Construction of glycosylation and phosphorylation site mutants

N-Glycosylation and tyrosine kinase phosphorylation site mutations were made with the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA). The AE2 protein sequence contains three potential *N*-glycosylation sites (consensus: Asn-X-Ser/Thr) in positions N859, N868 and N882. These asparagines were mutated to glutamines. The primer pairs designed for this purpose were: F859, 5′-CTGCTCAGCCTCCCAAAGCTCAGAGGTGG-3′ (nt 2562–2590) and R859, 5′-CCACCTCTGAGCTTTGGGAGGCTGAGCAG-3′; F868, 5′-ACGGCGGTGAGCAAATGACATGGGCC-3′ (nt 2591–2616) and R868, 5′-GGCCCATGTCATTTGCTCACCGCCGT-3′; F882, 5′-ACGCTGGGGCCGGGCCAAAGGAGCTTGG-3′ (nt 2629–2656) and R882, 5′-CCAAGCTCCTTTGGCCCGGCCCCAGCGT-3′ (F forward primer; R reverse primer; corresponding codons are underlined).

Two potential tyrosine kinase phosphorylation sites were identified in the AE2 sequence (residues 590 and 1154) using the ExPASy protein analysis program (Swiss Institute of Bioinformatics). The primer pairs used for mutating each of these tyrosines to asparagine (Y590N, F1136N, and Y1154N) were F590/R590 (5′-ACGAGGCAGCCAACCTGGCTGACG-3′/5′-CGTCAGCCAGGTTGGCTGCCTCGT-3′) and F1154/R1154 (5′-AGATGTCACTAACGTCAAGAAGG-3′/5′-CCTTCTTGACGTTAGTGACATCT-3′). We also mutated the tyrosine residue 1136 present in the –YERL– sequence (see Section 3), using the primer pair F1136/R1136 (5′-GGGATCCAGTTCAACGAGCGGCTGCAT-3′/5′-ATGCAGCCGCTCGTTGAACTGGATCCC-3′). All mutations were made according to the manufacturer's instructions.

2.5. Detergent extraction with Triton X-100

COS-7 and HeLa cells were cultured on coverslips to about 50–70% confluence. The cells were washed three times with PBS at 20°C and cooled on ice for 5 min. Ice-cold 1% Triton X-100 in TKM buffer (20 mM Tris, 10 mM KCl, 1 mM $MgCl_2$, pH 6.2) supplemented with protease inhibitors (Mini protease inhibitor tablets, Roche Diagnostics) was then added, and incubated for 30 min in an orbital shaker on ice. After detergent extraction, cells were rinsed briefly with PBS and fixed and stained prior to fluorescence microscopy. In control experiments, cells were fixed before the detergent extraction protocol.

2.6. Immunoblotting

Immunoblotting of endogenous AE2 protein was performed using native polyacrylamide gel electrophoresis (PAGE) gels, because the epitope recognized by the AE2 antibody has been shown to be sodium dodecyl sulfate (SDS)-sensitive [11]. Briefly, cells were washed two or three times with PBS and lysed directly on ice into 50 mM Tris, 150 mM NaCl, 1% Nonidet P-40, pH 8.0 [35] supplemented with protease inhibitors (Mini protease inhibitor tablets). After removal of insoluble material by centrifugation, 2×PAGE sample buffer was added, and the samples were then heated for 3 min at 94°C before subjection to PAGE using 4–12% Tris-glycine gradient gels (Novex). Samples were transferred onto a nitrocellulose filter and probed with the anti-AE2 antibody followed by peroxidase-conjugated secondary anti-rabbit antibodies (P.A.R.I.S.). The bands were visualized using the ECL detection system and Hyperfilm ECL (Amersham).

Immunoblotting with the anti-Ank$_{195}$ and anti-GFP antibodies was performed as follows: cells (untransfected and transfected) were washed two or three times with PBS and lysed directly with SDS–PAGE sample buffer supplemented with protease inhibitors and a reducing agent (5% mercaptoethanol). Alternatively, washed cells (on plates) were first subjected to detergent extraction (as described above, except that Triton X-100 was used instead of Nonidet P-40), prior to solubilization of the remaining insoluble material with the SDS sample buffer. After treatments, equal amounts of the Triton X-100- and SDS-solubilized samples were subjected to SDS–PAGE and immunoblotting with the anti-Ank$_{195}$- and anti-GFP antibodies. When applicable, DNA was sheared from the samples by vortexing (1500 rpm, 2 h).

2.7. Cell surface biotinylation

Biotinylation of GFP-AE2 transfected cells was performed as described by Li et al. [36]. Briefly, cells were treated on ice for 1 h with 0.5 mg/ml sulfo-NHS-LC-biotin (Pierce, Rockford, IL, USA) in borate buffer (10 mM boric acid, 154 mM NaCl, 7.2 mM KCl, 1.8 mM $CaCl_2$). After rinsing, cells were solubilized with a buffer containing 1% deoxycholate, 1% Triton X-100, 10 mM Tris, 150 mM NaCl and 1 mM EDTA (pH 7.5). After centrifugation, biotinylated proteins were absorbed from the supernatant using immobilized streptavidin beads (Sigma). Total, bound and unbound fractions were collected and subjected to SDS–PAGE before immunoblotting with the monoclonal anti-GFP antibody. The intensity of the bands was quantitated using the Scion image analysis program.

3. Results

3.1. Localization of the AE2 protein is cell type-dependent

Using the anti-AE2 C-terminal peptide antibody, we have

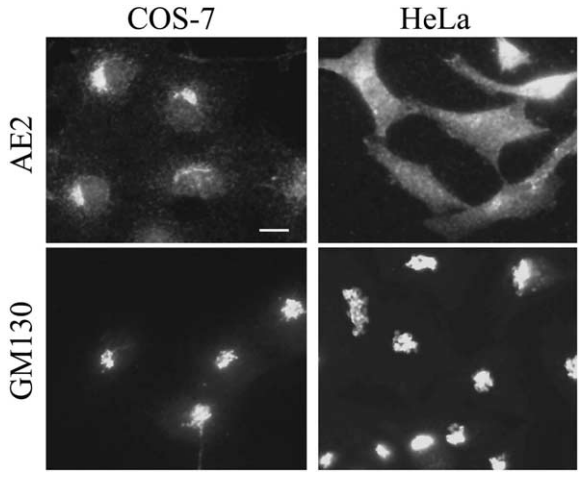

Fig. 1. Localization of the endogenous AE2 protein in COS-7 and HeLa cells. Cells grown on glass coverslips were stained either with the AE2 C-terminal peptide antibody (AE2), or with the anti-GM130 antibody (GM130, a Golgi marker). Note that the AE2 protein is localized mainly in the Golgi apparatus in COS-7 cells, whereas in HeLa cells the protein accumulates in the plasma membrane. Bar, 10 μm.

previously shown that the full-length AE2 protein is expressed in the Golgi membranes mainly in fibroblast-like cell types both in vivo and under normal cell culture conditions [26,27]. To assess first whether the endogenous AE2 protein is localized differently in epithelial cells, as suggested by the in vivo data [11–14], we screened a number of established cell lines with the same anti-AE2 antibody. During our initial screens, we found (Fig. 1) that in HeLa cells the staining pattern was clearly reminiscent of the plasma membrane staining, and not the characteristic Golgi staining pattern seen in COS-7 cells (Fig. 1). Both the lateral cell borders and the whole cell surface were stained, and intracellular or Golgi staining was undetectable. To allow comparisons, we stained COS-7 and HeLa cells with the Golgi marker antibody, GM130, and found (Fig. 1) that both cell types possessed a similar, compact juxtanuclear Golgi apparatus.

To confirm that the same full-length AE2 isoform is targeted differently between COS-7 and HeLa cells, we transiently expressed both the GFP- or DsRed-tagged AE2 fusion proteins and followed their distribution in these cells. As shown in Fig. 2, the GFP-tagged AE2 fusion protein localized, similarly to the endogenous AE protein, predominantly to the Golgi apparatus in COS-7 cells. In HeLa cells, the GFP-AE2 fusion protein did not accumulate in the Golgi region, but instead was detected mainly at the plasma membrane (Fig. 2). Transiently expressed DsRed-AE2 fusion protein gave identical results, i.e. it accumulated in the Golgi region in COS-7 cells and in the plasma membrane in HeLa cells (data not shown). Based on cell surface biotinylation, we estimated that 18.5% of the GFP-AE2 fusion protein was expressed at the cell surface in COS-7 cells, and 65.5% in HeLa cells. These values are fully consistent with our microscopical observations, which show that in addition to the Golgi region, a proportion of the GFP-AE2 fusion protein is detected in the plasma membrane in COS-7 cells, and in intracellular/Golgi membranes in HeLa cells. These results show that the full-length AE2 isoform is indeed targeted in a cell type-dependent manner mainly to the Golgi apparatus in fibroblast-like COS-7 cells and to the plasma membrane in epithelial HeLa cells.

3.2. Post-translational processing of the AE2 protein

To assess whether translational or post-translational processing differences between COS-7 and HeLa cells are responsible for their alternative targeting of the AE2, we first compared the molecular size of the endogenous AE2 protein expressed in these cells. Because the AE2 C-terminal epitope is sensitive to SDS [11], native gel electrophoresis system was used for this purpose. Immunoblotting with the anti-AE2 C-terminal antibody (Fig. 3A) showed that only one protein band was detected near the top of the native 4–12% PAGE gel in both COS-7 and HeLa cells. The bands had identical migration rates in this gel system, indicating that the AE2 proteins expressed by these cells do not markedly differ from each other.

Similar results were obtained when the GFP-tagged AE2 fusion protein was expressed in these cells. With the anti-GFP monoclonal antibody, we detected two different-sized bands in both cell types (Fig. 3B). The major, lower molecular

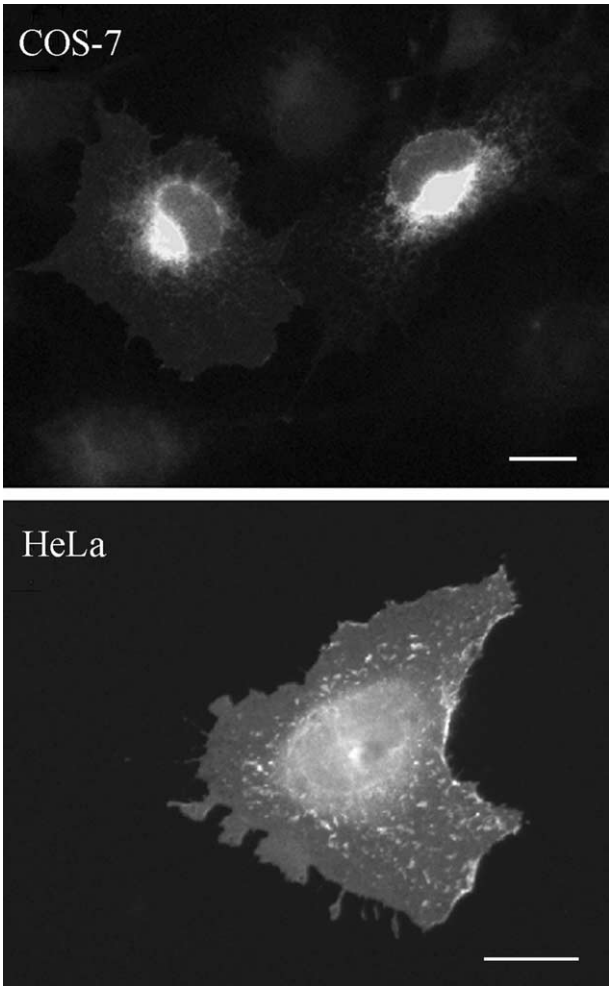

Fig. 2. Localization of the GFP-tagged full-length AE2 protein in COS-7 and HeLa cells. Cells were grown on glass coverslips, and transfected transiently with the plasmid encoding the full-length AE2 protein tagged N-terminally with the GFP protein. After 24 h, cells were fixed and examined by fluorescence microscopy. The DsRed-tagged full-length AE2 protein gave identical results (data not shown). Bars, 10 μm.

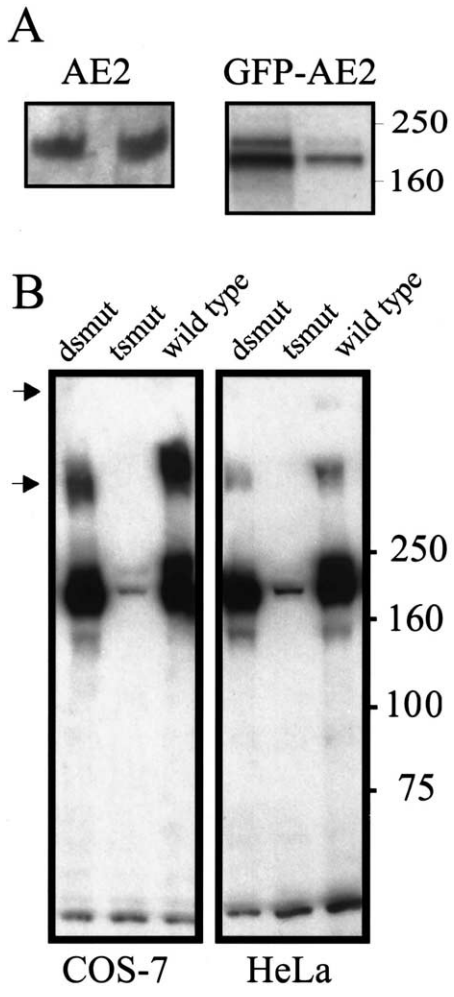

Fig. 3. Immunoblotting of the AE2 protein expressed in COS-7 and HeLa cells. A, Left: The endogenous AE2 protein was detected from total cell lysates with the C-terminal AE2 peptide antibody using native polyacrylamide gels (4–12% Novex). Note that in both cell types, only one major band of the same molecular size was detected with the antibody. Right: Immunoblotting of the expressed GFP-AE2 fusion protein in COS-7 and HeLa cells. The fusion protein was detected using the monoclonal anti-GFP antibody, which recognized identical fusion protein bands in both cell types. The faster migrating band represents the unglycosylated form of the fusion protein (195 kDa), whereas the upper band represents the fully glycosylated form of the fusion protein. B: Immunoblotting of the different *N*-glycosylation mutants of the GFP-AE2 fusion protein in COS-7 and HeLa cells. Note that in both cell lines, the molecular sizes of the mutated proteins (dsmut = N859+868Q; tsmut = N859+868+882Q) are lower than that of the wild type protein. Note also that each mutant has an identical size when expressed in either COS-7 or HeLa cells. The tsmut protein (which lacks all the potential glycosylation sites) represents the non-glycosylated form of the fusion protein. The high molecular weight bands (arrows) probably represent dimeric and tetrameric forms of the fusion protein.

weight band (about 190 kDa) probably represents unglycosylated AE2 protein, since this band was detected also in the presence of tunicamycin, a drug that inhibits *N*-linked glycosylation (data not shown, see also below). The higher molecular weight band (about 230 kDa) probably represents glycosylated GFP-AE2 fusion protein. These values correspond well to the calculated molecular size of the GFP-AE2 polypeptide chain (GFP, 27 kDa; AE2, 165 kDa). In addition to these monomeric protein bands, we often detected two other minor bands with much higher molecular weight in both cell types. These probably represent homodimeric and homotetrameric forms of the GFP-AE2 fusion protein (data not shown, see below).

To evaluate the glycosylation state of the expressed GFP-AE2 fusion protein in the two cell types, we sequentially mutated all three potential *N*-glycosylation sites (asparagine in positions 859, 868 and 882) in the AE2 sequence to glutamine, which has the same hydrophobicity and charge (Fig. 3B). Mutation of the first (N859Q) asparagine was not found to affect the molecular size or the localization of the fusion protein in either cell line (data not shown). However, an additional mutation of the next asparagine (N868Q; dsmut) reduced the molecular size of both the monomeric and oligomeric forms of the fusion protein similarly in both cell types. This mutant protein also accumulated in the endoplasmic reticulum in both cell types (data not shown). Finally, when the last asparagine was also mutated (N882Q, tsmut), in both cell types the triple mutant protein accumulated in the endoplasmic reticulum and migrated as a sharp band with a molecular weight of about 195 kDa (Fig. 3B), i.e. the calculated size of the complete GFP-AE2 polypeptide chain and the size of the band detected also in the presence of tunicamycin. This band therefore represents the unglycosylated form of the GFP-AE2 fusion protein. Collectively, these results indicate that the last two potential *N*-glycosylation sites in the AE2 sequence are used in both COS-7 and HeLa cells, and that major glycosylation differences do not exist between these two cell types. The results also suggest that although *N*-linked glycosylation appears not to be important for either the Golgi or the plasma membrane localization of the AE2 protein in these cells, it seems to be necessary for correct folding of the AE2 protein and for its transport out of the endoplasmic reticulum.

The first one of the potential tyrosine kinase phosphorylation sites found in the AE2 protein sequence is located within the KQFHEAAY sequence (amino acids 573–580), near the

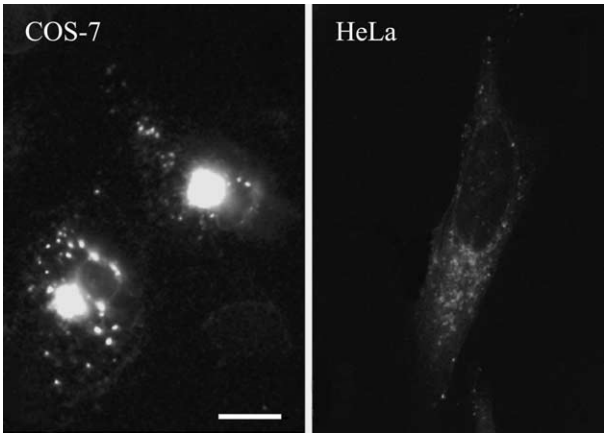

Fig. 4. Detergent solubility of the GFP-AE2 fusion protein in COS-7 and HeLa cells. Cells transiently transfected with the GFP-AE2 fusion protein-encoding plasmid were extracted with ice-cold Triton X-100, fixed and examined directly with a fluorescence microscope. Notice that the Golgi-localized AE2 protein in COS-7 cells resists detergent extraction in the cold, whereas no plasma membrane-associated GFP fluorescence can be detected in HeLa cells after detergent extraction. Only some residual fluorescence is present intracellularly in Triton X-100-treated HeLa cells. Bar, 10 μm.

first transmembrane domain. The other one is located within the last intracellular loop (KHHPDVTY sequence, amino acids 1147–1154). To exclude these two sites as potential effectors for targeting differences, we mutated these tyrosines to asparagines in both sequence motifs (Y580N, Y1154N). The mutations, however, did not affect the localization of the GFP-AE2 fusion protein in either cell type (data not shown). The AE2 C-terminal cytoplasmic tail also has a –YERL– sequence (residues 1136–1139) which is reminiscent of the well-known C-terminal tyrosine-based internalization motif (YQRL–) of the TGN38 protein [37]. Mutation of this tyrosine residue also did not change the distribution of the fusion protein in either COS-7 or HeLa cells (data not shown).

3.3. Detergent solubility of the expressed GFP-AE2 fusion protein

Proteins that either associate with cytoskeletal proteins, are present in lipid rafts [38,39] or form large oligomeric complexes [40], are characteristically resistant to Triton X-100 extraction in the cold. Therefore, we compared the detergent solubility of the Golgi- and the plasma membrane-localized GFP-AE2 fusion protein expressed in COS-7 and HeLa cells. We found, similarly to our earlier data [27], that a substantial

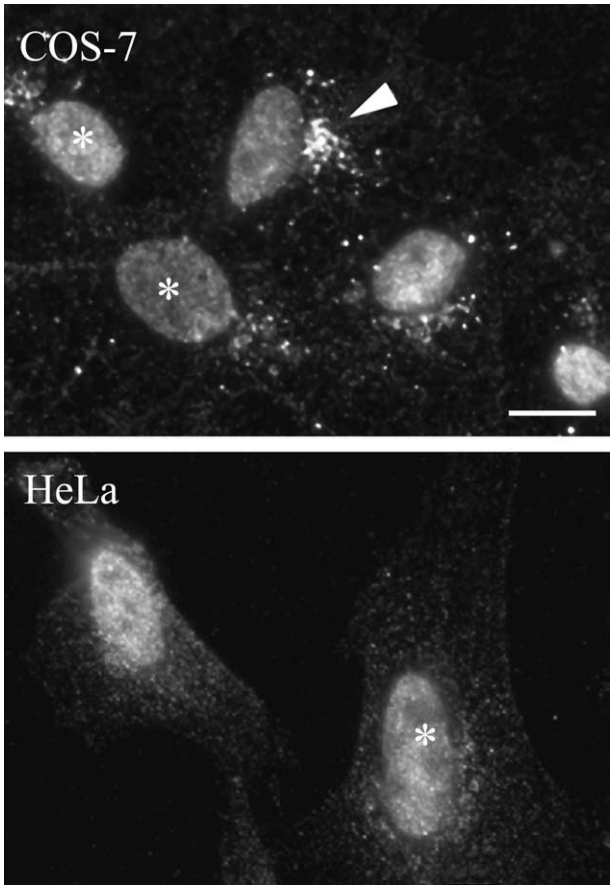

Fig. 5. Expression of Golgi-associated Ank_{195} in COS-7 and HeLa cells. Cells grown on glass coverslips were detergent-extracted, fixed and stained with the anti-Ank_{195} antibody. Indirect immunofluorescence shows staining of Golgi-associated Ank_{195} in COS-7 cells (arrowhead). In HeLa cells, the same antibody does not recognize a similar Golgi-like staining pattern. In both cell types, detergent extraction also resulted in staining of the nuclei (asterisks). Bar, 10 μm.

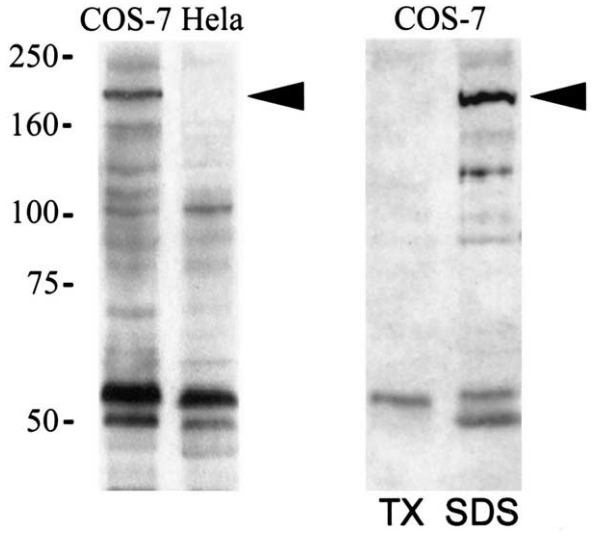

Fig. 6. Immunoblotting of Ank_{195} in COS-7 and HeLa cells. Left: Cells were solubilized directly in SDS sample buffer. Cleared total cell lysates were then subjected to SDS–PAGE before transfer onto a nitrocellulose filter. Immunoblot with the anti-Ank_{195} antibody shows a band with the apparent molecular size (193 kDa; arrowhead) of Ank_{195} protein in COS-7 cells, but not in HeLa cells. Right: Immunoblotting of COS-7 cells solubilized first with Triton X-100 (TX) and then with SDS. The blot shows that the 193 kDa band (arrowhead) is Triton X-100-insoluble.

proportion of the Golgi-localized AE2 fusion protein could not be solubilized with the Triton X-100 in intact cells (Fig. 4). In contrast, the plasma membrane-localized AE2 fusion protein appeared to be completely soluble, as evidenced by our inability to detect any cell surface-associated fluorescence in detergent-treated HeLa and COS-7 cells. Only some residual intracellular fluorescence was detected in HeLa cells after detergent extraction (Fig. 4). Prefixation of the cells prevented the solubilization of this plasma membrane-associated GFP-AE2 fusion protein (data not shown). Similar results were obtained with the DsRed-AE2 fusion protein in both cell types (data not shown).

3.4. Association of the AE2 fusion protein with the Golgi membrane skeleton

Based on its detergent resistance (Fig. 4), and the ability of some AE proteins to associate with ankyrins, we next considered the possibility that the Golgi-localized GFP-AE2 protein may be associated with either of the two ankyrin isoforms, AnkG119 or Ank_{195} [32–34] identified in the Golgi thus far. Of these two, the Ank_{195} isoform is the more probable candi-

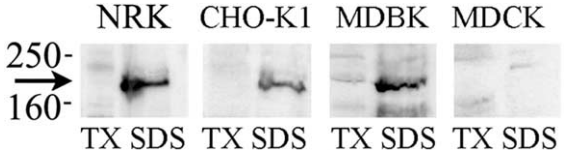

Fig. 7. Expression of the Ank_{195} in different cell lines. Cells grown on plates were sequentially extracted with Triton X-100 (TX) and SDS, as described in Section 2. Samples were then subjected to SDS–PAGE, and immunoblotted with the anti-Ank_{195} antibody. The blot shows the presence of the Triton X-100-insoluble 193 kDa band (arrow) in NRK, CHO-K1 and MDBK cells. Note also that MDCK cells do not express this Triton X-100-insoluble ankyrin isoform.

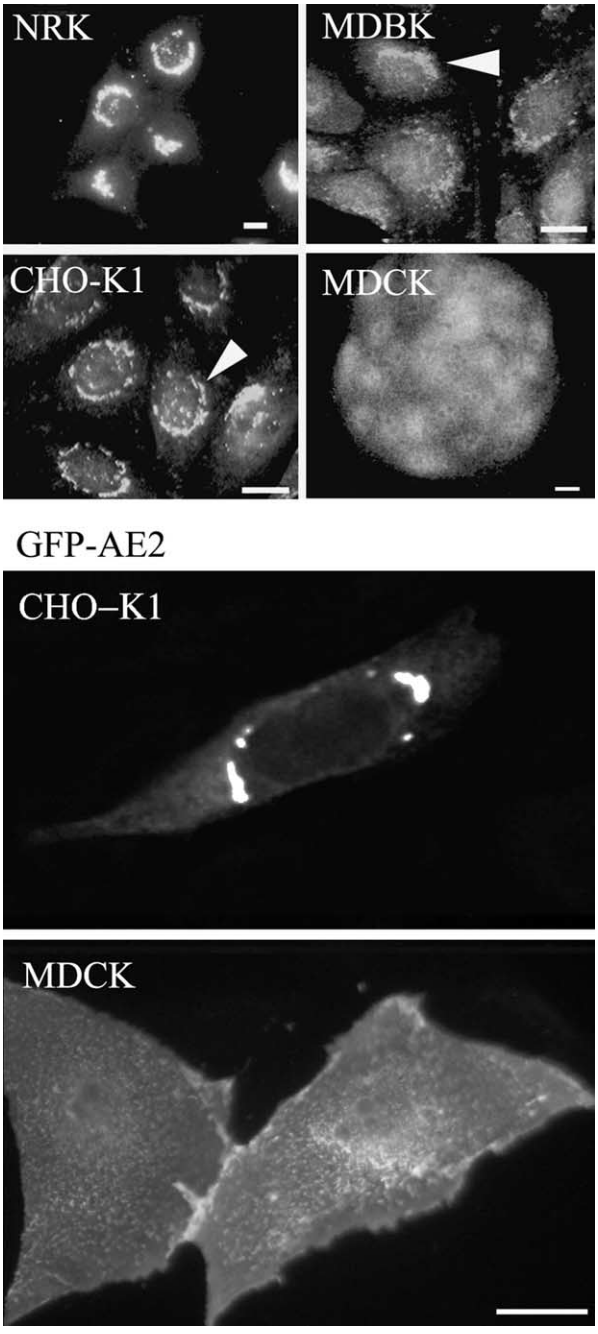

Fig. 8. Localization of the AE2 protein in NRK, CHO-K1, MDCK and MDBK cells. Top: Cells were grown on glass coverslips, fixed and stained with the anti-AE2 C-terminal antibodies. Note that the AE2 protein is localized in the Golgi membranes only in NRK, CHO-K1 and MDBK cells, which express the Ank_{195} isoform (compare with Fig. 7). In Ank_{195}-deficient MDCK cells, AE2 is localized predominantly to the plasma membrane. Bars, 10 μm. Bottom: Transient transfection of CHO-K1 and MDCK cells with the GFP-AE2 fusion protein-encoding plasmid. Note the localization of the fusion protein in the Golgi in Ank_{195}-expressing CHO-K1 cells, and in the plasma membrane in Ank_{195}-deficient MDCK cells.

date, as it is also resistant to Triton X-100 extraction [32] and like AE2 [26] it is concentrated in the *trans*-Golgi area [32]. Our initial cell stainings with the Ank_{195} antiserum revealed that it cross-reacts with several other ankyrin isoforms, as previously reported [32]. We, therefore, used detergent extraction to remove detergent-soluble ankyrin isoforms before staining of the cells. We found that in detergent-extracted COS-7 cells, the antiserum stained juxtanuclear Golgi-like structures, in addition to the nuclei (Fig. 5). However, in HeLa cells, despite the staining of the nuclei, no staining was observed in the juxtanuclear Golgi region, suggesting that HeLa cells do not express this ankyrin isoform. Our subsequent immunoblotting experiments with the same serum (Fig. 6) revealed a strong band of 193 kDa in COS-7 cells, but not in HeLa cells, thus confirming that COS-7 cells express the Ank_{195} isoform. Sequential fractionation of the cells with Triton X-100 and SDS revealed that this protein band in COS-7 cells is also Triton X-100-insoluble (Fig. 6).

Thus far, our attempts to directly demonstrate that the two proteins interact with each other have failed, mainly because the anti-Ank_{195} antiserum or the affinity-purified anti-AE2 antibodies do not work well for immunoprecipitation. To provide additional evidence for their potential interaction, we screened several other cell lines by immunoblotting and indirect immunofluorescence with the Ank_{195} antiserum and the anti-AE2 antibodies, respectively. As shown in Fig. 7, the Ank_{195} antiserum recognized a Triton X-100-insoluble 193 kDa band in most cell types including NRK, CHO-K1 and MDBK cells, but not in MDCK cells. Indirect immunofluorescence showed that the same cells also expressed the AE2 protein in the Golgi membranes (Fig. 8), whereas in Ank_{195}-deficient MDCK cells, the AE2 protein was localized to the plasma membrane. In addition, we succeeded in transiently transfecting two of these cell lines, CHO-K1 and MDCK cells, with the GFP-AE2 fusion protein-encoding plasmid, and found that the expressed fusion protein accumulated in the Golgi membranes in CHO-K1 cells, and in the plasma membrane in MDCK (Fig. 8). Jointly, these results together with the data from COS-7 and HeLa cells (Figs. 1 and 2) show that the localization of the AE2 protein in the Golgi and thus also its detergent resistance (Fig. 4) strictly correlate with the expression of Ank_{195} in the *trans*-Golgi.

Finally, we compared the subcellular distribution of the GFP-AE2 fusion protein and Ank_{195} in COS-7 and HeLa cells, after transient transfection of the cells with the GFP-AE2 fusion protein-encoding plasmid. Cells were stained with the Ank_{195} antiserum after detergent extraction. The results showed that the GFP-AE2 fusion protein and Ank_{195} indeed co-localize in the Golgi region in COS-7 cells but not in HeLa cells (Fig. 9). Both proteins were detected in the same highly compact juxtanuclear Golgi structure. In non-expressing cells (Fig. 9), Ank_{195} staining was more diffuse, suggesting that overexpression of the AE2 protein induces coalescence of Ank_{195} into AE2-enriched membranes. Such Golgi accumulation or GFP-AE2 coalescence was not observed in Ank_{195}-deficient HeLa cells.

4. Discussion

To better understand the variable subcellular distribution of the AE2 protein in vivo, we have explored here the possibility that the full-length AE2 protein is targeted to different subcellular membrane systems in a cell type-dependent manner. By using mainly fibroblast-like COS-7 cells and epithelial HeLa cells as target cells, we have shown that the endogenous full-length AE2 protein and its GFP- or DsRed-tagged variants are indeed targeted either to the Golgi apparatus in COS-7 cells or to the plasma membrane in HeLa cells. This

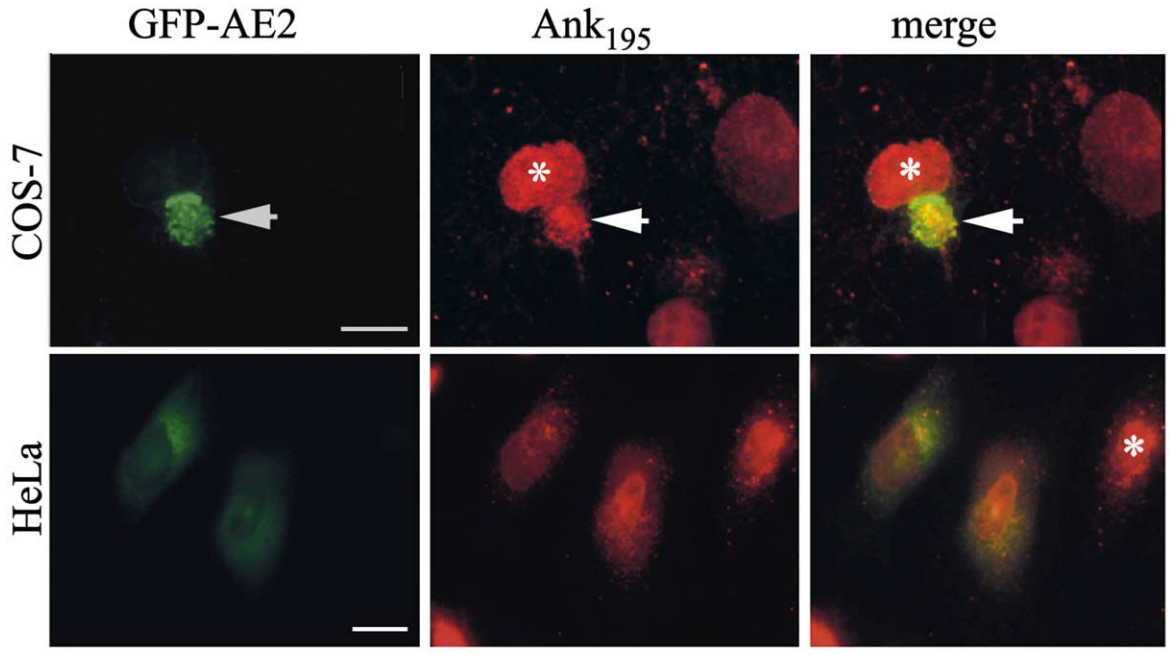

Fig. 9. Co-localization of the GFP-AE2 fusion protein and Ank_{195} in COS-7 cells. Cells transfected with the GFP-AE2 fusion protein construct were first extracted with Triton X-100, and then fixed and stained with the anti-Ank_{195} antibody. Note the co-localization and coalescence of the Ank_{195} and the GFP-AE2 fusion protein in the Golgi area in COS-7 cells (arrows), and the more disperse staining pattern of Ank_{195} in neighboring non-transfected cells. In detergent-extracted HeLa cells, however, no Golgi-localized AE2 or Ank_{195} is detectable. Note also the staining of the nuclei (asterisks) in detergent-extracted COS-7 and HeLa cells. Bars, 10 μm.

cell type-dependent targeting to the Golgi apparatus was the predominant one in most cells studied, and was found to correlate strictly with the expression of Ank_{195}, a *trans*-Golgi-associated membrane skeletal protein that has a high potential to interact with the AE2 protein. Although we failed to demonstrate their direct interaction in vivo, their similar detergent resistance, co-localization and coalescence, as well as the predominant localization of these two proteins on the *trans* side of the Golgi stack [26,32] all suggest that it is the direct interaction of these two proteins that is responsible for this cell type-dependent sorting of the AE2 protein in the Golgi. To our knowledge, this is the first description at the molecular level that targeting of this class of proteins does not seem to depend strictly on sequence-specific sorting motif(s) buried in the protein's primary structure. As such, our results may also explain at least some of the controversies that are related to the variant subcellular localization of the AE proteins in different tissues and cell types.

Cell type-dependent targeting mechanisms, as suggested by our data, do not preclude the existence of variant-specific targeting mechanisms that have been characterized recently mainly using chicken erythrocytes as a model system [9,10]. Rather, the suggested cell type-dependent mechanism can be regarded as an additional way to regulate the distribution of the AE2 protein, and perhaps also its homologues, between different subcellular membrane systems. It may be the predominant mechanism in mammalian cells, however. Interestingly, both mechanisms seem to utilize the same ankyrin-dependent localization mechanism, but in a different way. Variant-specific targeting has been shown to rely on alternative splicing and/or the use of alternative promoters [18], whereas the cell type-dependent mechanism suggested here appears to be based on the differential expression of Ank_{195} between cells.

Our findings together with the previous data described above emphasize an important role for ankyrins (and perhaps for other cytoskeletal/cytoplasmic proteins such as kanadaptin) as the main determinants for the variable localization of AE proteins in various tissues and cells in vivo. However, it is not known whether the potential interaction of these two proteins is itself sufficient for the localization of the AE proteins. Several Golgi-specific targeting mechanisms and signals have been put forward during the last decade. These include oligomerization, kin recognition, and the thickness of the lipid bilayer [40–43]. Given the roughly similar oligomerization states of the AE2 protein in COS-7 and HeLa cells, oligomerization itself appears not to be the primary cause for Golgi localization of the AE2 protein. Oligomerization may, however, indirectly affect localization, because ankyrin binding has been reported to be required for AE1 tetramerization [44]. In addition to our data (Fig. 3B) the ability of the AE2 protein to form oligomers has also been demonstrated with the use of cross-linking agents [45]. In this context, it should be noticed that our data also do not exclude the possibility that the plasma membrane-localized AE2 protein may associate with some distinct ankyrin isoform(s) that, however, have to be detergent-soluble based on our Triton X-100 solubility assays (Figs. 4 and 5). This view is also supported by the observed co-localization of the two proteins in the basolateral membrane of kidney epithelial cells [15] or gastric parietal cells [17] and the in vitro binding of the erythrocyte ankyrin to the N-terminus of AE2 [17].

A well-organized membrane skeleton in erythrocytes is known to be necessary for the structural integrity, mechanical stability and deformability of erythrocytes. The Golgi membrane skeleton has been suggested to have a similar membrane-stabilizing role in non-erythroid cells, perhaps by providing a platform for organized distribution of various

cytoplasmic and Golgi membrane proteins [46]. Golgi membrane skeletal proteins have also been suggested to facilitate efficient transport of some specific membrane proteins between the endoplasmic reticulum and the Golgi apparatus [47]. In either case, the AE2 protein may have a crucial role. First, the AE2 protein may have a pure structural role in the Golgi, and may provide a membrane anchorage site for the Golgi membrane skeletal proteins via Ank_{195}, perhaps in a phosphorylation-dependent manner [48]. Indeed, our recent data (Holappa et al., submitted) indicate that the AE2 protein is necessary for the structural integrity of the Golgi apparatus in COS-7 cells. The known ion transport properties of the AE2 protein in turn seem to provide an additional possibility to regulate Golgi pH and its pH-dependent functions, such as glycosylation, protein sorting and efficient vesicular trafficking through this organelle. The predominant (but not exclusive) expression of the AE2 protein at the plasma membrane in HeLa and MDCK cells is also not in contrast with either of the two possibilities, and may in fact reflect a specific need of some epithelial cell types to express the AE2 anion exchanger at the plasma membrane.

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