Regions of human kidney anion exchanger 1 (kAE1) required for basolateral targeting of kAE1 in polarised kidney cells: mis-targeting explains dominant renal tubular acidosis (dRTA)

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Accepted 11 November 2003
Journal of Cell Science 117, 1399-1410 Published by The Company of Biologists 2004
doi:10.1242/jcs.00974

Summary

Distal renal tubular acidosis (dRTA) is characterised by defective acid secretion by kidney α-intercalated cells. Some dominantly inherited forms of dRTA result from anion exchanger 1 (AE1) mutations. We have developed a stably transfected cell model for the expression of human kidney AE1 (kAE1) and mutant kAE1 proteins in MDCKI cells. Normal kAE1 was delivered to the plasma membrane of non-polarised cells and to the basolateral membrane of polarised cells. The AE1 N-glycan was processed to a complex form. Surprisingly, expression of kAE1 increased the permeability of the paracellular barrier of polarised MDCKI monolayers. All dominant dRTA mutations examined altered the targeting of kAE1 in MDCKI cells. The mutant proteins kAE1(R589H), kAE1(S613F) and kAE1(R901Stop) were retained in the ER in non-polarised cells, but the kAE1(R901Stop) protein was also present in late endosomes/lysosomes. The complex N-glycan of kAE1(R901Stop) was larger than that of normal kAE1. In polarised cells, the mutant kAE1(R901Stop) was mis-targeted to the apical membrane, while the kAE1(R589H) and kAE1(S613F) mutants did not reach the cell surface. These results demonstrate that dominant dRTA mutations cause aberrant targeting of kAE1 in polarised kidney cells and provide an explanation for the origin of dominant dRTA. Our data also demonstrate that the 11 C-terminal residues of kAE1 contain a tyrosine-dependent basolateral targeting signal that is not recognised by μ1B-containing AP-1 adaptor complexes. In the absence of the N-terminus of kAE1, the C-terminus was not sufficient to localise kAE1 to the basolateral membrane. These results suggest that a determinant within the kAE1 N-terminus co-operates with the C-terminus for kAE1 basolateral localisation.

Key words: Renal tubular acidosis, Basolateral protein Targeting, Kidney AE1 anion exchanger

Introduction

The human erythrocyte anion transport protein, the anion exchanger 1 (AE1) (also known as Red Blood Cell protein Band 3) carries out chloride-bicarbonate (Cl^-/HCO_3^-) exchange in the red blood cell and in the distal nephron of the kidney (reviewed by Tanner, 2002). The human kidney isoform (kAE1) is identical to AE1 but lacks the N-terminal 65 amino acids of AE1 (Kollert-Jons et al., 1993). kAE1 is located in the basolateral membrane of kidney α-intercalated cells (Alper et al., 1989; Drenckhahn et al.,1985; Schuster et al., 1986) and functions in acid secretion into the urine. An apical H^+-ATPase pumps protons derived from hydrated CO_2 into the tubular lumen. The HCO_3^- remaining in the cells is moved into the blood by kAE1 in exchange for chloride.

Distal renal tubular acidosis (dRTA) is characterised by defective acid secretion by the α-intercalated cells. All the reported dominantly inherited forms of the disease result from mutations in the AE1 gene (Bruce et al., 1997; Bruce et al., 2000; Jarolim et al., 1998; Karet et al., 1998). These dominant dRTA mutations include several that affect residue R589 (R589H, R589S, R589C) located in the intracellular loop between transmembrane span (TMS) six and seven (Bruce et al., 1997; Jarolim et al., 1998; Karet et al., 1998). Other mutations are S613F in TMS seven (Bruce et al., 1997), A858D in the final TMS (Bruce et al., 2000) and an 11 amino acid deletion in the C-terminus (R901Stop, previously known as band 3 Walton) (Karet et al., 1998; Toye et al., 2002). The most prevalent and thus the most clinically significant inherited forms of dRTA are associated with alterations of R589 of the AE1.

Mutant AE1 proteins of dominant dRTA retain substantial anion transport activity in the mutant red blood cells and also when these kAE1 proteins are expressed in *Xenopus* oocytes (Bruce et al., 1997; Bruce et al., 2000; Toye et al., 2002). Furthermore, the presence of one transport-inactive AE1 allele in simple heterozygotes (such as in Southeast Asian ovalocytosis) does not give rise to dRTA (Bruce et al., 2000). This indicates that dominant dRTA does not result from a simple reduction in anion transport activity of the mutant kAE1, because a single normal kAE1 allele can provide adequate acid secretion by the intercalated cells (Bruce et al., 1997; Bruce et al., 2000). Alternative mechanisms suggested for the dominant disease include mis-targeting of the mutant

protein to the apical membrane and/or altered targeting of the normal protein by interaction with the mutant protein (Bruce et al., 1997; Jarolim et al., 1998).

We recently demonstrated that, the kAE1 protein was delivered to the cell surface when expressed in non-polarised stably transfected Madin-Darby canine kidney cells type I (MDCKI) cells, whereas the kAE1(R901Stop) mutant did not reach the cell surface (Toye et al., 2002). Other transient transfection studies also showed that kAE1 with R589H or R901Stop mutations is retained internally in HEK293 cells and that each of the mutant kAE1 proteins can hetero-oligomerise with the normal kAE1 protein (Quilty et al., 2002a; Quilty et al., 2002b). However, to fully characterise the effects of these mutations on kAE1 in the kidney and understand the basis of the kidney disease, it is important to investigate these mutations using a model system in which polarised targeting can be observed.

In this paper, we demonstrate the basolateral targeting of normal kAE1 in stably transfected polarised MDCKI cell cultures. We also show that the expression of normal kAE1 in polarised MDCKI monolayers increases the permeability of the paracellular barrier. Importantly, all three dominant dRTA kAE1 mutant proteins traffic abnormally in this system. Protein kAE1 with R589H or S613F mutations does not reach the surface of the polarised cells, whereas the kAE1(R901Stop) mutant is mis-targeted to the apical instead of the basolateral membrane. This mis-targeting of the kAE1(R901Stop) mutant from the basolateral to the apical membrane is consistent with the removal of information required for basolateral localisation. The C-terminal amino acids deleted in the kAE1(R901Stop) mutant contain both a tyrosine motif and a type-II PDZ-interaction domain. We showed that the tyrosine residue, but not the PDZ domain, is crucial for basolateral localisation. The kAE1 was also delivered to the basolateral membrane in LLC-PK1 cells, suggesting that kAE1 does not require the μ1B-containing AP-1 adaptor complex for basolateral trafficking. Furthermore, in the absence of the N-terminus of kAE1, the C-terminus was not sufficient to localise kAE1 to the basolateral membrane. These results suggest that a determinant within the kAE1 N-terminus co-operates with the C-terminus for the basolateral localisation of kAE1.

Materials and Methods

Antibodies

Bric 170 and Bric 155 recognise epitopes of kAE1 at the N- and C-termini, respectively (Wainwright et al., 1989). Bric 6 reacts with an extracellular kAE1 epitope (Smythe et al., 1995). The above are mouse monoclonal antibodies. Rabbit anti-TGN38 was available in-house (Wilde et al., 1992). Rabbit anti-LAMP1, anti-COPI and anti-COPII antibodies were kindly provided by H. Mellor and D. Stephens (University of Bristol, UK). Rabbit anti-calnexin (StressGen, San Diego, CA), anti-early endosomal antigen (EEA1) antibody (Affinity Bioreagents, Golden, CO), rat anti-ZO1 (Biogenesis, Poole, UK), chicken anti- Na^+/K^+-ATPase (AbCam.com, Cambridge, UK) were used. Other antibodies used were: Donkey anti-rat TRITC antibody (Research Diagnostics, Flanders, NJ), TRITC conjugated goat anti-chicken antibody (Serotec, Oxford, UK), goat anti-mouse TRITC and anti-rabbit FITC secondary antibodies (Stratec Scientific, Cambridgeshire, UK), biotin-conjugated anti-mouse antibody and FITC-labelled anti-biotin antibody (Vector Labs, Burlingame, CA) .

Construction of cDNAs encoding kAE1 mutants

The plasmids BSXG.KB3, BSXG.KB3R589H, BSXG.KB3S613F BSXG.KB3R901Stop (previously denoted BSXG.KB3Walton) and BSXG.B3mem have been described (Bruce et al., 1997; Toye et al., 2002; Groves et al., 1996), and comprise the coding sequence of normal kAE1 and kAE1(R589H), kAE1(S613F), kAE1(R901Stop) and AE1mem in the expression vector BSXG. The normal kAE1 cDNA was used to make C-terminal mutant constructs with Y904A and V907A [kAE1(Y904A,V907A)], Y904F [kAE1(Y904F)], an V907A [kAE1(V907A)] and the deletion of the C-terminal V (kAE1V911Stop) by PCR. The coding sequences were TOPO-TA cloned into pcDNA3 vector as directed by the manufacturer (Invitrogen, Paisley, UK). All constructs were confirmed by DNA sequencing.

Cell transfection

Stably-transfected MDCKI cells were obtained as described previously (Toye et al., 2002). Cells were polarised by either seeding them at high density on coverslips or by seeding them onto permeable filters (Nunc, FisherScientificUK, Leicestershire, UK). LLC-PK1 cells (gift from I. Mellman, Yale, USA) were grown as described (Folsch et al., 1999). LLC-PK1 cells were transfected using Fugene-6 reagent™ following the manufacturer's instructions (Roche, Mannheim, Germany).

Immunofluorescence microscopy

Cells were processed for confocal microscopy as described previously (Toye et al., 2002). For antibody double labelling of cells for kAE1 with intracellular markers, rabbit anti-calnexin, anti-TGN38, anti-LAMP1, anti-COPI, anti-COPII or anti-EEA1 in 4% BSA was applied after incubation with anti-AE1 and followed by compatible anti-mouse TRITC and anti-rabbit FITC secondary antibodies. Cells probed for LAMP-1 were incubated with 0.05% saponin (Sigma, Poole, UK) and then 3% paraformaldehyde and all subsequent steps were done in 0.01% saponin. For optimal images of kAE1 expressed in polarised MDCKI cells, the cells were treated with 5 mM sodium butyrate for 12 hours and incubated at 27°C for 4 hours. Detection of kAE1 was further improved by utilising a biotin-conjugated anti-mouse antibody and FITC-labelled anti-biotin antibody. Fluorescence imaging was done using a Leica TCS-NT confocal laser scanning microscope (Leica-Microsystems, Milton Keynes, UK) equipped with a Kr-Ar laser. For dual-labelling confocal microscopy studies, FITC or TRITC images were taken sequentially with excitation-emission filters set at short pass 510 nm/band pass 530 nm for FITC and band pass 568 nm/long pass 590 nm for TRITC.

Labelling of cell surface AE1 with anti-AE1 antibody

FITC-conjugated BRIC 6 (FITC-Bric 6) anti-AE1 antibody was applied directly to washed intact unpolarised cells on coverslips for 30 minutes at 37°C. Washed cells were fixed with methanol at –20°C for 5 minutes and imaged by confocal microscopy. For cells polarised on permeable filters, intact cell layers were washed with ice-cold PBS and incubated at 4°C for 15 minutes. FITC-Bric 6 was applied to apical and basolateral surfaces and cells were incubated at room temperature for 1 hour, washed, fixed and imaged as above.

[^{35}S] methionine metabolic labeling of cells and immunoprecipitation

MDCKI cells stably transfected with kAE1, kAE1 variants or the vector alone, were incubated with 5 mM sodium butyrate for approximately 12 hours and then incubated for 30 minutes in methionine-free DMEM (Sigma) at 37°C. The cells were then labelled with 200 μCi/ml of Easy Tag™ [^{35}S]-L-methionine (NEN Life

Science Products, London, UK) in methionine-free DMEM. After labelling the medium was removed and replaced with DMEM containing 10% fetal calf serum (FCS). Cells were lysed in 2% SDS in immunoprecipitation buffer (IP buffer) [which contained 150 mM NaCl, 6 mM EDTA, 50 mM Tris-HCl (pH 7.5), 200 μM phenylmethanesulfonylfluoride (PMSF), 10 μg/ml aprotinin, and 5 μg/ml each of leupeptin, pepstatin A and antipain], and then 20 volumes 1% Triton X-100, 1% sodium deoxycholate, 1% BSA, 0.2% FCS in IP buffer was added. After pre-clearing with protein A-agarose beads (Bio-Rad, Hertfordshire, UK), the lysate was incubated overnight at 4°C with Protein A-agarose beads preloaded with either Bric 155 or Bric 170 plus rabbit anti-mouse IgG antibody (Dako). The beads were washed with IP buffer containing 1% Triton X-100, 1% sodium deoxycholate and 0.1% sodium dodecyl sulfate (SDS), the immune complexes were separated by SDS-PAGE and the labelled proteins detected by fluorography.

Cell surface biotinylation

Cell surface biotinylation was carried out on metabolically labelled cells as described by Li et al. (Li et al., 2000). Cells were treated with non-penetrating biotinylation reagent EZ-link™ Sulfo-NHS-biotin (Pierce, Rockford, IL) at 4°C for 30 minutes, total kAE1 was then immunoprecipitated and the fraction of biotinylated kAE1 determined by binding to streptavidin beads. For domain specific biotinylation of polarised cells grown on permeable filters, the non-penetrating biotinylation reagent was added at 4°C for 30 minutes on the apical or basolateral surface and then processed as above. To image biotinylated monolayers, non-labelled cells on filters were biotinylated for 30 minutes at 4°C on the apical surface. Cells were then washed and fixed with methanol or methanol/acetone (6:4 v/v) (for kAE1 colocalisation) at –20°C for 5 minutes. Cells were blocked with 4% BSA for 15 minutes and biotinylated proteins were detected with a goat anti-biotin antibody.

Endoglycosidase digestion of immunoprecipitates

Immunoprecipitates from [^{35}S]methionine-labelled cells were eluted from the protein A beads by boiling in SDS-PAGE sample buffer for 30 seconds followed by adding 2 volumes of 2.5% NP40 (w/v) in 125 mM sodium phosphate buffer (pH 7.5). Equal aliquots of the sample were not digested or digested with endoglycosidase H (Endo H) or N-glycanase F (PNGase F) (New England Biolabs, Beverly, MA).

Results

Stable expression of normal and mutant kAE1 in a kidney cell line

We have previously described transfected MDCKI cell clones that stably express normal kAE1 (MDCKI-kAE1 cells) (Toye et al., 2002). MDCKI canine renal epithelial cells form tight polarised monolayers with electrical and biochemical properties resembling those of collecting duct cells (Richardson et al., 1981). We found no evidence for endogenous kAE1 in MDCKI cells by immunofluorescence or immunoblot analysis using anti-AE1 antibodies that recognise AE1 in canine red blood cell membranes (A.M.T., G.B., M.J.A.T., unpublished data). Confocal microscopy studies of MDCKI-kAE1 cells with anti-AE1 antibodies showed expression of kAE1 predominantly at the plasma membrane, with some intracellular staining. To locate the intracellular population of kAE1 in these non-polarised cells, we performed dual-label immunofluorescence analysis with a range of organelle-specific antibodies (see Materials and Methods). We observed a significant overlap only between kAE1 and COPI, kAE1 and TGN38 (A.M.T., G.B., M.J.A.T., unpublished data), suggesting that the majority of the intracellular pool of kAE1 in non-polarised MDCKI cells occurs in the later stages of the secretory pathway.

We also made different MDCKI cell lines stably expressing mutated kAE1 with mutations associated with dominant dRTA (R589H, S613F or R901Stop); here referred to as, for example, MDCKI-kAE1(R589H). Mutant proteins kAE1(R589H) and kAE1(S613F) failed to reach the cell surface in unpolarised MDCKI-kAE1(R589H) and MDCKI-kAE1(S613F) cells (Fig.1). Only a small number of these stably transfected cells contained detectable amounts of the kAE1 mutants as determined by confocal microscopy. The kAE1(R589H) and kAE1(S613F) mutant proteins localised predominantly with calnexin, an endoplasmic reticulum (ER) marker (Fig. 1C,F). There was very little colocalisation with TGN38 or COPI

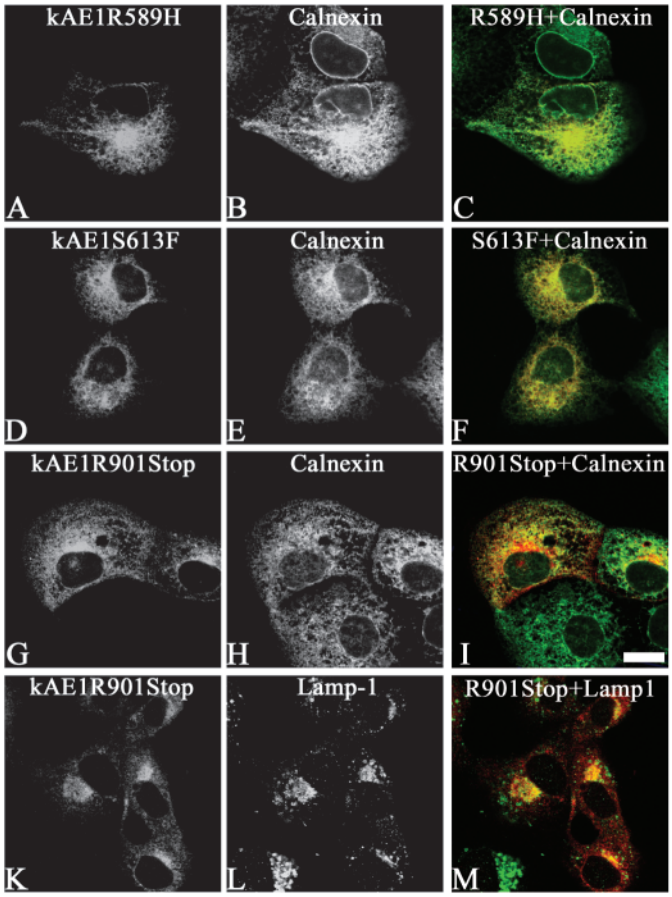


Fig. 1. Colocalisation of kAE1(R589H), kAE1(S613F) and kAE1(R901Stop) proteins with intracellular markers in non-polarised MDCKI cells. (A-C) MDCKI-kAE1(R589H), (D-F) MDCKI-kAE1(S613F) and (G-M) MDCKI-kAE1(R901Stop). Cells were treated with Bric 170 anti-AE1, followed by anti-calnexin or anti-LAMP1 antibodies and then incubated with TRITC-conjugated anti-mouse secondary antibody and FITC-conjugated anti-rabbit secondary antibody. (A,D,G,K) Visualising anti-AE1. (B,E,H) Visualising anti-calnexin. (L) Visualising anti-LAMP1. (C,F) Merged images show calnexin and kAE1(R589H) and kAE1(S613F) staining (overlaps in yellow). (I,M) Merged images show that kAE1(R901Stop) staining partially overlaps with calnexin (I) and that kAE1(R901Stop) also localises to a perinuclear region that overlaps with LAMP1 (M). Scale bar, 15 μm.

(A.M.T., G.B., M.J.A.T., unpublished data), suggesting that the mutant proteins were being retained within the ER. Similar colocalisation with calnexin was reported for kAE1(R589H) in transiently transfected HEK293 cells (Quilty et al., 2002a).

The mutant protein kAE1(R901Stop) was retained within the MDCKI cells as previously observed (Fig. 1G) (Toye et al., 2002). Double antibody labelling showed that kAE1(R901Stop) partially colocalised with calnexin (Fig. 1I). However, an intracellular pool of the mutant kAE1(R901Stop) also colocalised with LAMP-1, a late endosomal and/or lysosomal marker (Fig. 1M), suggesting that although kAE1(R901Stop) does not reach the plasma membrane, it exits the ER and mis-targets to late endosomes and/or lysosomes.

Incubation of the cells at a lower temperature (27°C), which corrects CFTR(ΔF508) mis-trafficking in transfected cells (Denning et al., 1992), did not alter the subcellular distribution of kAE1 carrying the R589H, S613F or R901Stop mutations (A.M.T., G.B., M.J.A.T., unpublished data).

FITC-conjugated Bric 6 (FITC-Bric 6) recognises an extracellular epitope on kAE1 and was used to assess the delivery of the normal and mutant forms of kAE1 to the cell surface. After incubating FITC-Bric 6 with intact kAE1-expressing cells, we found that FITC-Bric 6 was bound to the surface of cells expressing normal kAE1 (Fig. 2D) but not the surface of cells transfected with empty vector (Fig. 2B), confirming that the normal kAE1 reaches the cell surface. FITC-Bric 6 failed to bind to intact MDCKI-kAE1(R589H) or MDCKI-kAE1(S613F) cells, even after overnight incubation with sodium butyrate to increase transgene expression from cytomegalovirus (CMV) promotor and incubation at 27°C (Gorman and Howard, 1983; Denning et al., 1992). This is consistent with the observation that these mutant proteins are trapped in the ER. The vast majority of MDCKI-kAE1(R901Stop) cells also did not bind FITC-Bric 6 (Fig. 2F). However, a minority of the MDCKI-kAE1(R901Stop) cells (2.8%; n=642**)** bound a small amount of FITC-Bric 6 (Fig. 2H), and this proportion of cells increased following sodium butyrate treatment (6.6%; n=602) (A.M.T., G.B., M.J.A.T., unpublished data).

Cell surface biotinylation was also used to determine the amount of cell surface expression of kAE1 and kAE1(R901Stop) in non-polarised MDCKI cells at steady state. The percentage of the total kAE1 present at the cell surface (±s.d.) was 51±6% for kAE1 (n=3) and 11±3% for kAE1(R901Stop) (n=3). These data support the results obtained by using FITC-Bric 6 (Fig. 2) and confirm that much less kAE1(R901Stop) reaches the cell surface in non-polarised MDCKI cells. Interestingly, the proportion of total kAE1 present at the cell surface after incubation in the presence of sodium butyrate was reduced to 20±3% for kAE1 (n=6) and 5±2% for kAE1(R901Stop) (n=6). This difference between cells incubated in the presence and absence of sodium butyrate correlates with the observation that sodium butyrate treatment increases kAE1 expression, and indicates that the increased kAE1 expression results in less efficient delivery of the protein to the cell surface. This is true for normal kAE1 and kAE1(R901Stop).

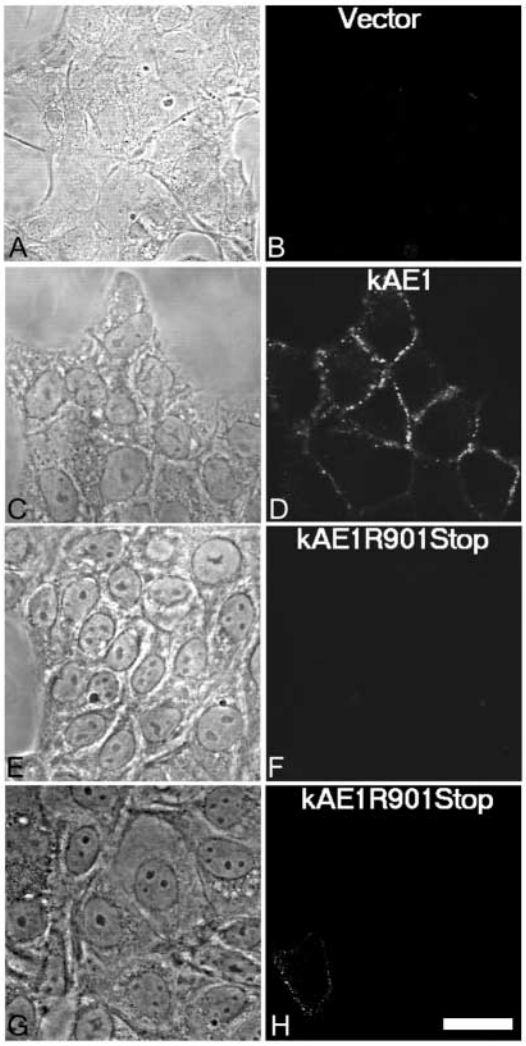


Fig. 2. Surface labelling of non-polarised MDCKI-kAE1 cells with FITC-Bric 6 anti-AE1 antibody. MDCKI cells stably expressing p3.1 vector (A,B), kAE1 (C,D) or kAE1(R901Stop) (E-H) were incubated with FITC-Bric 6, which recognises an extracellular epitope on AE1. (A,C,E,G) bright field images. (B,D,F,H) fluorescence images. The majority of kAE1 cells bind FITC-Bric 6 (D), whereas the majority of kAE1(R901Stop) cells do not bind Bric 6, (F) with only a small number of obvious positive cells (H). Scale bar, 15 μm.

Expression of normal and mutant kAE1s in polarised MDCKI cells

When the stable MDCKI-kAE1 cell clones were seeded at high density and allowed to polarise on coverslips or permeable filters kAE1 localised to the basolateral membrane (Fig. 3A). After seeding cells at high density, the expression of kAE1 decreased with time possibly because of silencing of the CMV promoter of the pcDNA3 vector (results not shown). This was partially reversed by incubating the cells with sodium butyrate and by following the strategies outlined in Materials and Methods. Polarised cells were double labelled with anti-kAE1 and anti-ZO-1 (which defines the cytosolic face of tight junctions) and anti-kAE1 with anti-Na^+/K^+-ATPase (a basolateral membrane marker). Location of kAE1 was on the lateral membrane below the tight junction marker ZO-1 (Fig. 3B) and together with Na^+/K^+-ATPase at the basolateral membrane (Fig. 3C). The application of FITC-Bric 6 antibody to both apical and

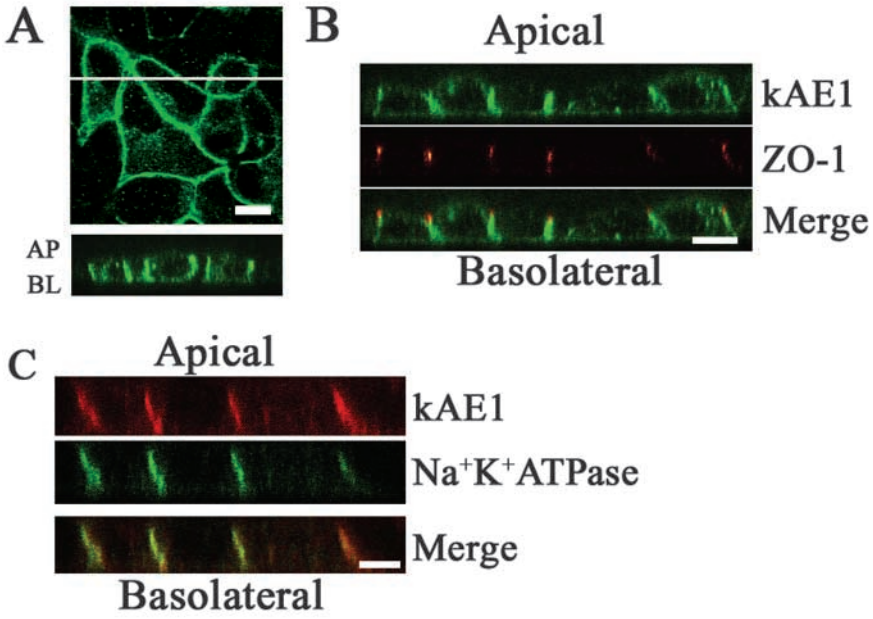


Fig. 3. In polarised MDCKI cells, kAE1 is localised to the basolateral membrane. MDCKI cells stably expressing kAE1 were seeded at high density and allowed to polarise. (A) Cells were incubated with anti-AE1 Bric 170, then a biotin-conjugated anti-mouse antibody and visualised using a FITC-labelled anti-biotin antibody. The upper and lower images show focal planes parallel and perpendicular to the cell monolayer, respectively. (B,C) MDCKI-kAE1 cells were double-stained with anti-AE1-Bric 170, and either a tight junction marker anti-ZO-1 antibody (B) or an anti-Na^+/K^+ATPase antibody (C). Merged images show kAE1 located on the lateral side of the ZO-1 (B) and colocalises with the basolateral marker Na^+/K^+-ATPase (C). Scale bars, 10 μm. AP, apical; BL, basolateral.

basolateral surfaces of intact MDCKI-kAE1 cells that were polarised on filters also resulted in exclusively basolateral binding of the antibody (Fig. 4D), demonstrating that normal kAE1 is expressed at the surface of the basolateral membrane of the cells.

Because the kAE1 mutants associated with dRTA fail to reach the surface of non-polarised MDCKI cells, we asked whether similar trafficking defects are apparent in stably transfected but polarised cells. As observed with MDCKI-kAE1, polarisation reduced the number of cells expressing detectable amounts of mutant kAE1 proteins (A.M.T., G.B., M.J.A.T., unpublished data). Mutants kAE1(R589H) and kAE1(S613F) were distributed throughout the polarised cells and did not reach the cell surface (Fig. 4A,B). Also, these cells did not bind FITC-Bric 6 when the antibody was applied to apical and basolateral surfaces (A.M.T., G.B., M.J.A.T., unpublished data), showing that both proteins [kAE1(R589H) and kAE1(S613F)] do not reach the plasma membrane of polarised cells. By contrast, protein kAE1(R901Stop) localised to the apical membrane in polarised MDCKI cells (Fig. 4C). Apical localisation was also observed in the absence of sodium butyrate (A.M.T., G.B., M.J.A.T., unpublished data), suggesting that this localisation is not because of overexpressed kAE1(R901Stop) protein, overwhelming the cellular sorting machinery. Furthermore, the application of FITC-Bric 6 to apical and basolateral surfaces of intact MDCKI-kAE1(R901Stop) cells that had been polarised on filters, resulted in an exclusively apical binding of the antibody (Fig. 4E) and confirms that kAE1(R901Stop) localises to the apical surface.

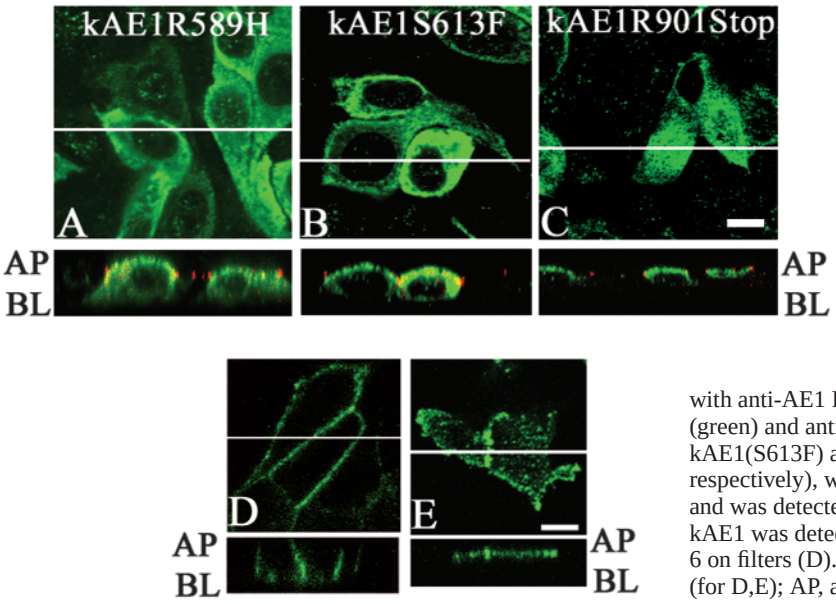


Fig. 4. Polarised expression of dominant dRTA kAE1 mutants. MDCKI-kAE1(R589H) cells (A), MDCKI-kAE1(S613F) cells (B), MDCKI-kAE1(R901Stop) cells (C,E) and MDCK1-kAE1 cells (D) were polarised on filters. (A-C) Cells were stained for AE1 using Bric 170 (green), (A-C, bottom panels) tight junctions were localised with anti-ZO-1 antibody (red). (D,E) FITC-Bric 6 antibody was added to apical and basolateral surfaces of the intact cell monolayer. (Top panels) Focal planes taken parallel to the epithelium near the centre of the cells (A,B,D) and at the apical surface (C,E). (Bottom panels) Focal planes perpendicular to the epithelium. (A-C, top) Staining with anti-AE1 Bric 170; (A-C, bottom) merged images of anti-AE1 (green) and anti-ZO-1 staining (red). Proteins kAE1(R589H) and kAE1(S613F) are both distributed throughout the polarised cells (A,B, respectively), whereas kAE1(R901Stop) is at the apical membrane (C) and was detected exclusively at the apical surface by FITC-Bric 6 (E). kAE1 was detected exclusively at the basolateral surface by FITC-Bric 6 on filters (D). Scale bar in C, 12 μm (for A-C); scale bar in E, 15 μm (for D,E); AP, apical; BL, basolateral.

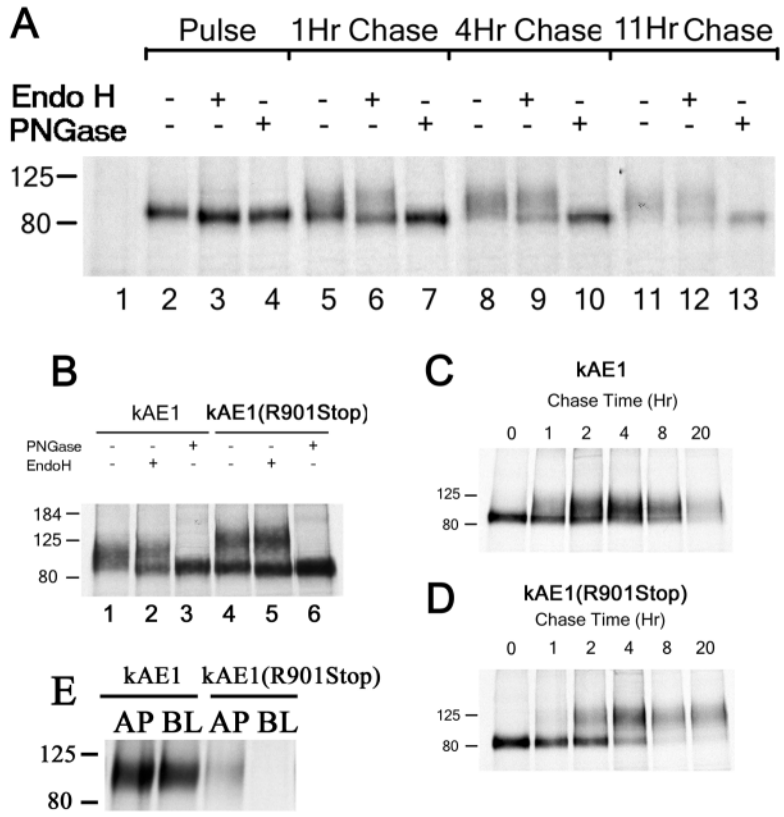


Fig. 5. Radiolabelling of kAE1 and kAE1(R901Stop) in polarised (A,E) or non-polarised (B,C,D) MDCKI cells. Cells were preincubated with sodium butyrate and labelled with 200 μCi/ml Easy-Tag™ [^{35}S]methionine for 30 minutes (A,C,D) and pulse chased, or steady-state labelled for 4 hours (B,E). Immunoprecipitates were prepared using anti-AE1 Bric 155 (A) or Bric 170 (B-E) as described in Materials and Methods. Immunoprecipitates were treated with endo H or PNGase F where indicated and analysed by SDS-PAGE. (A) shows a pulse-chase experiment on MDCKI-kAE1 cells polarised on filters. The p3.1 vector control is shown in lane 1. (B) Steady-state labelled non-polarised MDCKI-kAE1 and MDCKI-kAE1(R901Stop) cells, respectively. (C,D) Pulse chase studies on non-polarised MDCKI-kAE1 cells (C) or MDCKI-kAE1(R901Stop) cells (D). (E) Selective biotinylation of the apical or basolateral surfaces of kAE1 and kAE1(R901Stop) cells polarised on filters. The apical and basolateral surface was selectively biotinylated, total AE1 immunoprecipitated and the biotinylated fraction selected using streptavidin beads. AP, apical; BL, basolateral.

Complex N-glycosylation of kAE1 and kAE1(R901Stop) in MDCKI cells

AE1 contains a single N-glycosylation site at N642. Pulse-chase experiments were carried out on polarised MDCKI-kAE1 cells (Fig. 5A) labelled with [^{35}S]methionine. Labelled kAE1 was then immunoprecipitated and its susceptibility to endoglycosidases was examined. At the start of the pulse chase (Fig. 5A, lane 2), kAE1 manifests as a single band of ~93kDa that is both endo H and PNGase F sensitive (lanes 3 and 4, respectively), indicating that the kAE1 N-glycan is predominantly of the high mannose type at this point. Within 1 hour, an additional diffuse upper band was observed that was endo H-insensitive (lane 6) but PNGase F-sensitive (lane 7). Over time, this diffuse upper band, which contains the complex form of N-glycan, increased in intensity whereas the endo H-sensitive band intensity decreased, an observation correlating with the passage of kAE1 through the secretory pathway. Similar results were obtained when non-polarised MDCKI-kAE1 cells were used (Fig. 5C). We were unable to achieve sufficient radioactive labelling of kAE1 in polarised MDCKI-kAE1(R901Stop) cells to obtain corresponding pulse-labelling data, probably because of the low number of cells expressing the mutant protein after polarisation.

We compared the protein expression of normal kAE1 and kAE1(R901Stop) in [^{35}S]methionine-labelled non-polarised MDCKI cells. The kAE1(R901Stop) mutant was expressed at a level similar to normal kAE1 in non-polarised cells (Fig. 5B). Protein kAE1(R901Stop) was present as a form of higher molecular weight than kAE1 (Fig. 5B, compare lane 1 with lane 4) and was insensitive to endo H (lane 5) but sensitive to PNGase F (lane 6), and thus contains a complex N-glycan. Therefore, kAE1(R901Stop), like normal kAE1, receives modifications to its N-glycan that normally occur in or beyond the medial Golgi. This is consistent with the immunofluorescence localisation data shown in Fig. 1. The complex N-glycan of kAE1(R901Stop) is larger than that of normal kAE1 because it migrates more slowly on SDS-PAGE, even though the kAE1(R901Stop) polypeptide is 11 amino acids shorter (Fig. 5B, compare lanes 1, 2 with lanes 4, 5). This might indicate a longer transit time of kAE1(R901Stop) through the medial Golgi, an increased recycle process of the mutant protein, or different trafficking compared with normal kAE1.

Pulse-chase metabolic labelling was carried out in non-polarised MDCKI cells expressing normal kAE1 (Fig. 5C) and kAE1(R901Stop) (Fig. 5D). No intermediate form (of a size corresponding to the normal complex N-glycan) was observed on the kAE1(R901Stop) mutant before the formation of the larger complex N-glycan. The similar amounts of normal kAE1 and kAE1(R901Stop) after 20 hours of pulse-chase indicate that this mutant protein was as stable as normal kAE1 in the cells (Fig. 5, compare C with D).

The expression levels of kAE1 in all the MDCKI-kAE1(R589H) and MDCKI-kAE1(S613F) cell clones were too low to visualise by using radioactive methionine labelling, even in non-polarised cells after treatment with sodium butyrate and incubation at 27°C (A.M.T., G.B., M.J.A.T., unpublished data). This is consistent with the small proportion of cells that contain detectable amounts of the kAE1 mutants as assessed by confocal microscopy (A.M.T., G.B., M.J.A.T., unpublished data). Neither the proteosome inhibitor lactacystin, nor the lysosomal proteinase inhibitor leupeptin increased the number of cells expressing detectable levels of the recombinant proteins (A.M.T., G.B., M.J.A.T., unpublished data).

Domain selective cell surface biotinylation was carried out in polarised MDCKI cells that expressed normal kAE1 and kAE1(R901Stop) and were grown on permeable filters. Under these conditions, kAE1 was accessible to the impermeant biotinylation reagent from both the apical and basolateral

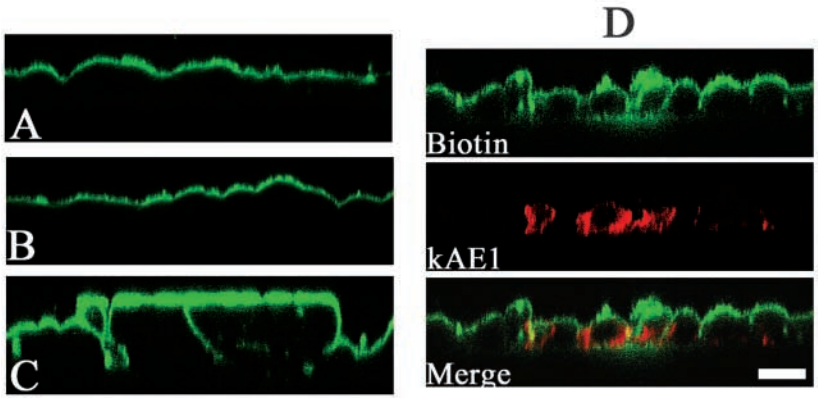


Fig. 6. Polarised MDCKI cells expressing kAE1 are permeable to biotin reagent. MDCKI (A), MDCKI-kAE1(R901Stop) (B) and MDCKI-kAE1 (C) cells were selectively biotinylated on the apical surface. Biotinylated proteins were detected using a FITC-conjugated anti-biotin antibody. (D) MDCKI-kAE1 cells stained for biotinylated proteins (green, top panel) and kAE1 (red, middle panel). kAE1 cells have lateral staining (C, top panel D) suggesting that they are permeable to the usually impermeant biotinylation reagent. Polarised MDCKI-kAE1 cells biotinylated on the lateral membranes (green) clearly correspond to cells expressing kAE1 (red) (D, bottom panel). Scale bar, 15 μm

surfaces (Fig. 5E). This conflicted with the results of the immunochemical staining, which demonstrated a uniquely basolateral localisation for kAE1. Although barely detectable, the kAE1(R901Stop) mutant was labelled only at the apical surface, entirely consistent with the confocal imaging data. To establish the reason for the discrepancy between the immunocytochemical data and the biotinylation results for normal kAE1, polarised MDCKI, MDCKI-kAE1 and MDCKI-kAE1(R901Stop) cells were all biotinylated at the apical surface under the same conditions. The biotinylated regions were visualised with a FITC-conjugated anti-biotin antibody. Biotinylation was restricted to the apical surfaces of polarised monolayers of MDCKI or MDCKI-kAE1(R901Stop) cells (Fig. 6A,B). By contrast, 'patches' of laterally stained cells were observed among the polarised MDCKI-kAE1 cells (Fig. 6C and D, top). Double staining of these monolayers for the biotin reagent and anti-kAE1 showed that these 'patches' of cells with lateral staining for biotin were also expressing kAE1 (Fig. 6D, bottom). Furthermore, while the transepithelial electrical resistance of polarised untransfected MDCKI cells or kAE1(R901Stop) MDCKI cells grown on permeable filters rose to 9000-13,000 Ω/cm during polarisation, the transepithelial resistance of MDCKI-kAE1 cells only rose to half this value (4000-6000 Ω/cm), even though tight junctions (defined by ZO-1 staining) were still present. The increased penetration of the usually membrane-impermeant biotinylation reagent through the monolayers indicates that expression of kAE1 at the basolateral membrane of polarised MDCKI cells increases the permeability of the paracellular barrier, rather than enhancing a permeability pathway through the cells themselves. This reconciles the differences observed between the immunocytochemical and biotinylation results.

kAE1 Basolateral sorting determinants

The aberrant localisation of kAE1(R901Stop) compared with normal kAE1 in polarised MDCKI cells, clearly suggests that the 11 residues at the C-terminal end of kAE1 (–RDEYDEVAMPV) contain a basolateral targeting or retention determinant. The C-terminus contains two motifs that are absent in the mutant kAE1(R901Stop) and that might target kAE1 to the basolateral membrane: a potential tyrosine-based sorting motif YDEV (conforming to YxxΦ where x is any amino acid and Φ is a hydrophobic amino acid) and a class-II PDZ-interacting domain (AMPV) (Cowan et al., 2000).

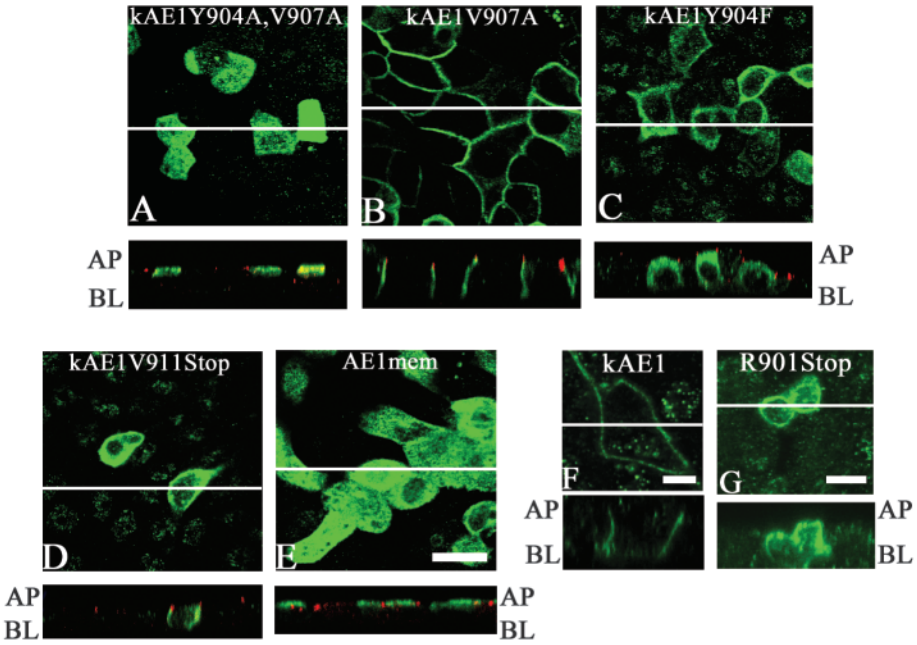
C-terminal mutations were constructed in kAE1 to determine the role of the potential targeting motifs in AE1 basolateral localisation. Stable MDCKI transfectants expressing these kAE1 mutants were generated. YxxΦ sequences associate with the μ subunit of adaptor complexes by binding a region of the μ subunit containing pockets for Y and Φ residues (Owen and Evans, 1998). Therefore, we created the double mutant kAE1(Y904AV907A), in which tyrosine and valine of the YDEV sequence were substituted with alanine. In polarised cells, kAE1(Y904AV907A), like the kAE1(R901Stop) mutant, is mis-targeted to the apical membrane (Fig. 7A). We also created single substitution mutants of V907A [kAE1(V907A)] and Y904F [kAE1(Y904F)] to assess the importance of these individual residues. The kAE1(V907A) mutant did not change kAE1 basolateral localisation in polarised cells (Fig. 7B). Either V907 is not crucial for the basolateral targeting of kAE1 or the targeting machinery can tolerate the substitution of valine with alanine at this position. However, the mutation Y904F altered kAE1 localisation and resulted in the intracellular retention of the mutant kAE1 (Fig. 7C). Cells expressing kAE1(Y904F) did not bind FITC-Bric 6 when the antibody was applied to apical and basolateral surfaces, showing that this mutant does not reach the plasma membrane of polarised cells (A.M.T., G.B., M.J.A.T., unpublished data). This contrasts with the apical localisation of the mutants kAE1(R901Stop) and kAE1(Y904A,V907A) and suggests that the single Y904F mutation alters kAE1 trafficking in a different way. Taken together, these data confirm that Y904 is essential for the basolateral targeting of kAE1.

The PDZ-domain-interacting sequence remained unaltered in the above mutants even though some mutants were mis-targeted. Thus the PDZ-binding sequence is not sufficient to target kAE1 to the basolateral membrane. To abolish PDZ domain interactions (Silver, 2002), we also constructed a kAE1 mutant, in which the last valine residue is deleted [kAE1(V911Stop)]. This mutant was localised to the plasma membrane in non-polarised cells (A.M.T., G.B., M.J.A.T., unpublished data) and targeted basolaterally in polarised MDCKI cells (Fig. 7D). Mutant kAE1(V911Stop) was localised to the basolateral membrane, and kAE1(V911Stop)-transfected cells contained more intracellular kAE1 protein than cells transfected with normal kAE1 (compare Figs 3A and 4D with Fig. 7D). These results suggest that although the PDZ-interacting domain is not essential for basolateral localisation,

it might be required for more a efficient trafficking to, or for retention at the basolateral surface.

A comparison of the C-terminal sequence of AE1 in different species illustrates that a tyrosine residue is present in human, mouse, rat, bovine, chicken and trout (Fig. 7H). Chicken kAE1 has two basolateral localisation determinants in the N-terminus that are absent in the human kAE1 (Adair-Kirk et al., 1999). When these N-terminal determinants are removed, chicken kAE1 localises to the apical membrane even though the C-terminal tyrosine motif remains. We explored the importance of the human kAE1 N-terminus by generating a MDCKI stable cell line expressing the AE1 membrane domain (AE1mem) (residues 361-911 of AE1) that lacks the N-terminal domain but retains the C-terminus (Groves et al., 1996). Unlike kAE1(R901Stop), AE1mem was efficiently localised to the plasma membrane in non polarised cells (A.M.T., G.B., M.J.A.T., unpublished data). Surprisingly, the AE1mem construct was also localised to the apical membrane of polarised cells (Fig. 7E). Therefore an element within the N-terminal sequence is required in addition to the YxxΦ motif of the C-terminus for the basolateral targeting of kAE1.

The μ1B subunit of the AP-1 adaptor interacts with tyrosine based sorting signals and can mediate basolateral sorting (Folsch et al., 1999). LLC-PK1 cells, which lack the μ1B subunit, missort basolateral membrane proteins and carry μ1B-dependent tyrosine-based sorting signals to the apical membrane (Folsch et al., 1999). LLC-PK1 cells were transiently transfected with kAE1 and were allowed to polarise. The kAE1 protein was targeted exclusively to the basolateral membrane in the polarised LLC-PK1 cells, suggesting that μ1B is not necessary for basolateral sorting of this protein (Fig. 7F). The transient expression of kAE1(R901Stop) in polarised LLC-PK1 cells resulted in both apical and basolateral distribution of the protein (Fig. 7G).



H

Human	AE1	-GRDEYDEVAMPV-COOH
Mouse	AE1	-GLDEYDEVPMPV-COOH
Rat	AE1	-GLDEYDEVPMPV-COOH
Taurus	AE1	-GQDEYDEVAMPV-COOH
Chicken	AE1	-GQDVYNEVQMPS-COOH
Trout	AE1	-GEDMYESPLP---COOH

Fig. 7. Effects of kAE1 C-terminal mutations and the deletion of the kAE1 N-terminus in polarised MDCKI and LLC-PKI cells. Filter-polarised cells MDCKI-kAE1 (Y904A,V907A) (A), MDCKI-kAE1(V907A) (B), MDCKI-kAE1(Y904F) (C), MDCKI-kAE1(V911Stop) (D) and MDCKI-AE1mem (E) were stained with Bric 170 for AE1. Tight junctions were stained with ZO-1 antibody. (B-D, top panels) Focal planes parallel to the epithelium near the centre of the cells. (A,E, top panels) Focal planes at the apical surface. (All bottom panels) Focal planes perpendicular to the epithelium. (A-E, top and middle panels) Staining with anti-AE1 Bric 170. (A-E, bottom panels) Merged images of anti-AE1 (green) and anti-ZO-1 staining (red). The double mutant kAE1(Y904AV907A) and also kAE1Y904F were mis-targeted in MDCKI cells (A and C, respectively) whereas kAE1(V907A) had identical localisation to normal kAE1 (B). This suggests that the tyrosine residue at position 904 is crucial for basolateral targeting of kAE1. Removal of the N-terminus in the construct AE1mem also resulted in the mis-targeting of kAE1 to the apical membrane. (F,G) Polarised LLC-PK1 cells transiently transfected with normal kAE1 (F) or kAE1(R901Stop) (G). Cells were stained for AE1 using Bric 170. localisation of kAE1 was basolateral in LLC-PK1 cells, while kAE1(R901Stop) showed apical and basolateral location. (H) Sequence alignment of C-termini of AE1 from different species. Scale bars in E, 20 μm (for A-E), in F and G, 10 μm. AP, apical; BL, basolateral.

Discussion

Human kAE1 has a basolateral location in kidney α-intercalated cells (Alper et al., 1989; Drenckhahn et al., 1985) that is essential for its function in acid secretion. The study of human kAE1 trafficking and the role of kAE1 mutations in dRTA, therefore required the availability of a polarised cell model. To address this, we have developed a stably transfected cell model for the expression of kAE1 and kAE1 mutant proteins in polarised MDCKI cells. We show that mutations in dominant dRTA cause aberrant targeting of kAE1. These dRTA mutations highlight regions within the kAE1, which are essential for the correct localisation of the protein in kidney cells.

kAE1 Expression in MDCKI cells

Wild type kAE1 was localised to the basolateral membrane of polarised, stably transfected, MDCKI cells. This mirrors the basolateral localisation of kAE1 in human kidney α-intercalated cells. We also show that human kAE1 is

progressively converted from a core glycosylated form to a complex glycosylated form in both polarised and non-polarised MDCKI-kAE1 cells. Similar processing to a complex N-glycan was observed on chicken kAE1 in MDCK cells (Adair-Kirk et al., 1999). These results contrast with those obtained using human kAE1 transiently transfected into HEK-293 cells, where processing of normal kAE1 and kAE1(R901Stop) did not proceed beyond the core N-glycan (Li et al., 2000; Quilty et al., 2002a; Quilty et al., 2002b). This probably reflects intrinsic differences in trafficking of kAE1 in HEK293 cells and MDCKI cells.

kAE1 affects the permeability of polarised MDCKI monolayers

Domain selective biotinylation is a widely accepted technique for ascertaining the surface membrane domain that proteins are delivered to in polarised cells. Surprisingly, we found that polarised monolayers of MDCKI cells that expressed wild-type kAE1 were leaky to the impermeant biotinylation reagent, although the localisation of the tight junction marker ZO-1 appeared to be unaffected. These cells also had lower transepithelial electrical resistance than control cells, another indication of increased paracellular permeability. This was observed only in cells transfected with normal kAE1 and not the kAE1(R901Stop) mutant, which suggests that disruption of the paracellular barrier either involves the C-terminal residues of kAE1 or requires kAE1 to be localised at the basolateral membrane. The decrease in transepithelial electrical resistance is small, suggesting a localised failure of a modest number of compromised cells rather than a global failure of the entire paracellular barrier. This is entirely consistent with the presence of small ‘patches’ of kAE1-expressing cells in polarised MDCKI-kAE1 cell monolayers, where groups of kAE1 positive cells are surrounded by cells with no detectable kAE1. The paracellular permeability can be altered in several ways, including perturbation of the actin cytoskeleton (which also leaves ZO-1 localisation unaffected) (Stevenson and Begg, 1994; Takakuwa et al., 2000). Overexpression of kAE1might interfere with the actin-spectrin cytoskeleton and affect the organisation of the paracellular permeability barrier provided by epithelial tight junctions. Further work will be necessary to elucidate the mechanism of this effect.

Trafficking of kAE1(R589H) and kAE1(S613F) proteins

The kAE1(R589H) and the kAE1(S613F) dRTA mutants were internally retained in non-polarised and polarised MDCKI cells, with no evidence for cell surface delivery of either protein. Protein kAE1(R589H) was transiently expressed in non-polarised HEK293 cells and also did not reach the plasma membrane (Quilty et al., 2002a). Mutations of R589 are associated with the most prevalent and clinically most significant form of dRTA and the localisation of this protein and the kAE1(S613F) mutant in polarised cells has not been reported previously. Both, R589H and S613F, mutations affect the region around TMSs six and seven of AE1 and they probably act in a similar fashion. The mutations do not cause gross misfolding of the mutant proteins because they still have significant transport activity in both *Xenopus* oocytes and red blood cells (Bruce et al., 1997; Jarolim et al., 1998). However, TMSs six and seven do not appear to be essential for the anion transport function of AE1 (Groves et al., 1998). Localised misfolding in the region around TMSs six and seven might increase the aggregation of the mutant proteins (thus affecting their exit from the ER (Ellgaard and Helenius, 2003) Alternatively, it might lead to mutant proteins being retained by chaperones and delivered to the degradation machinery or to the endoplasmic reticulum associated degradation system (ERAD).

Trafficking of kAE1(R901Stop) protein

This mutant protein had distinctly different cellular locations in polarised and non-polarised MDCKI cells, which also differed from the locations of normal kAE1 and the kAE1(R589H) and kAE1(S613F) mutants. In non-polarised cells the majority of kAE1(R901Stop) was internally retained as judged by surface antibody labelling and biotinylation. Pulse-chase studies on non-polarised MDCKI cells suggest that kAE1(R901Stop) leaves the ER, passes through the Golgi compartment (probably more slowly than normal kAE1 or by a different route, as hinted at by the aberrant glycosylation profile) and is retained in a post-Golgi compartment. The observation that normal kAE1 and kAE1(R901Stop) both exhibit similar half lives suggests that this compartment is not lysosomal. The larger than normal size of the complex N-glycan on the kAE1(R901Stop) protein might also indicate that this mutant recycles between the late endosomes and TGN, because it is unable to reach the plasma membrane.

Mutant protein kAE1(R901Stop) was localised to the apical membrane in polarised MDCKI cells. Transient expression of kAE1(R901Stop) in polarised MDCK cells has been recently reported, but Devonald et al. found that the protein had a non-polarised distribution, with the mutant protein present at basolateral and apical membranes (Devonald et al., 2003). This clearly differs from the purely apical localisation observed in our studies and might be due to different kAE1 expression levels in stably and transiently transfected MDCK cells. In support of this, we found that transient expression of kAE1(R901Stop) in polarised LLC-PK1 cells also resulted in both apical and basolateral distribution of the protein (Fig. 7G).

The different localisation of normal kAE1 and kAE1(R901Stop) in non-polarised and polarised MDCKI cells, clearly show that the 11 residues at the C-terminal end of kAE1 contain both a plasma membrane targeting determinant (in non-polarised cells) and a basolateral targeting or retention determinant (in polarised cells). The loss of this plasma-membrane basolateral signal could allow previously cryptic signals in the normal kAE1 protein to become dominant. It is also possible that a different hierarchy of signals is involved in non-polarised and polarised cell states. This could explain the different locations of kAE1(R901Stop) in non-polarised and polarised cells. For example, within the cytoplasmic loop of kAE1 (between TMSs six and seven) is a tyrosine based sequence (YFPGK-) that is similar to the lysosomal targeting signal YFPQA found in the third cytoplasmic loop of the lysosomal protein cystinosin (Cherqui et al., 2001) and which might influence kAE1 targeting in non-polarised cells. Furthermore, the altered, more complex N-glycan observed on kAE1(R901Stop) could potentially act as an apical targeting signal. This is because in the absence of a basolateral targeting

determinant, N-linked glycans can act as an apical targeting signals (Benting et al., 1999; Gut et al., 1998; Rodriguez-Boulan et al., 1999) in polarised cells. Further work is required to identify the signals that contribute to the abnormal targeting of kAE1 when the C-terminus is absent.

Mechanisms leading to dominant dRTA associated with kAE1 mutations

All three mutations associated with dominant dRTA that were examined in this study altered the targeting of kAE1 in MDCKI cells. Any attempts to explain the mechanism of dominant dRTA need to describe how the mutations cause the disease even though the patients carry a normal AE1 allele as well as the mutant allele. The mutant kAE1 could be mis-targeted to the apical membrane, as was reported for a variant LDL receptor in familial hypercholesterolemia (Koivisto et al., 2001). In addition, the mutant kAE1 could alter the targeting of normal kAE1 as a result of hetero-oligomerisation of the two proteins. This occurs in autosomal dominant *diabetes insipidus*, where the mutant aquaporin-2 mis-targets the wild-type protein by forming mixed oligomers (Asai et al., 2003; Marr et al., 2002). It has already been demonstrated that both the kAE1(R589H) and the kAE1(R901Stop) proteins can form hetero-oligomers with normal kAE1 when co-transfected in HEK293 cells (Quilty et al., 2002a; Quilty et al., 2002b).

Our study suggests that dRTA can arise by both mechanisms described above. The R589H mutation (and very likely the S613F mutation) probably causes the intracellular retention of the normal kAE1 by associating with the mutant protein. The R901Stop mutation mis-targets the protein to the apical membrane. The mis-targeted Cl^-/HCO_3^--exchanger at the apical membrane would allow HCO_3^- to move into the tubular lumen together with protons secreted by the H^+-ATPase, thus negating net acid secretion by the cells. In addition, the kAE1(R901) might misroute the normal protein by forming hetero-oligomers with it (Quilty et al., 2002b). Finally, it also possible that the abnormal localisation of active kAE1 affects intercalated cell function in other ways, for example by perturbing the pH in intercellular compartments. Experiments using co-transfection of normal kAE1 with the mutant kAE1s in the polarised MDCKI cell system would have defined more conclusively the molecular basis of the dRTA associated with each kAE1 mutation. Unfortunately, our attempts to do this using transient transfection were unsuccessful. In particular, we have so far been unable to observe polarised co-expression even when cDNA for epitope-tagged mutant kAE1 was transiently transfected into stable cell lines expressing normal kAE1 carrying a different epitope tag. Therefore, in order to investigate further the effects of co-expression of dRTA mutants on normal kAE1 we are now pursuing a dual expression stable cell model system.

Basolateral sorting signals in kAE1

Many normally basolateral membrane proteins are localised to the apical membrane after the removal of their basolateral targeting signals (Matter and Mellman, 1994). We were surprised to find that kAE1 has a requirement for two basolateral determinants, one in the N-terminus and one in the C-terminus. Unusually, the N- and C-terminal determinants are both necessary for basolateral localisation.

Basolateral targeting determinants often rely on tyrosine or dileucine-dependent motifs. The sequence Y^{904}DEV within the C-terminus conforms to the consensus motif YxxΦ, which binds to medium-chain subunits of adaptor complexes involved in various clathrin dependent and independent trafficking events (Aguilar et al., 2001; Boehm and Bonifacino, 2001; Dell'Angelica et al., 1997; Ohno et al., 1996; Stephens and Banting, 1998). The Y(904)DEV motif is similar to YMEV, one of two motifs required for basolateral targeting of aquaporin- 4 (Madrid et al., 2001). The potential tyrosine-based sorting signal in kAE1 is adjacent to a type-II PDZ-binding motif, which was shown to bind PICK-1 (Cowan et al., 2000). PDZ-binding-domain proteins are widely expressed, are often involved in targeting and regulation of membrane proteins (Sheng and Sala, 2001) and can retain apical or basolateral membrane proteins in their respective domains (Moyer et al., 1999; Olsen et al., 2002). However, we have shown that Y904, but not the C-terminal PDZ-binding motif, is absolutely required for the basolateral localisation of kAE1. The importance of Y904 was recently confirmed independently by transiently expressing epitope-tagged kAE1Y904A in MDCK cells (Devonald et al., 2003). Although not essential for kidney basolateral targeting, increased intracellular kAE1 levels were observed in polarised MDCKI cells when the PDZ-interaction domain was truncated in the kAE1V911stop mutant. This suggests that this PDZ motif might still play some role in the efficient trafficking and/or retention at the basolateral membrane.

The mutation of Val to Ala at position Y+3 in the YxxΦ motif in kAE1 still resulted in basolateral sorting, whereas a valine mutation at Y+3 changed the basolateral localisation of aquaporin-4, suggesting that, even aquaporin-4 and kAE1 share a similar tyrosine motif, they probably interact with different targeting components. Alanine is present at Y+3 in the major basolateral tyrosine sorting motif of the Na^+/taurocholate co-transporter (Sun et al., 2001). This motif does not conform to the standard YxxΦ targeting motif and Sun et al. suggest that it does not interact with a clathrin-adaptor complex for basolateral targeting (Sun et al., 2001). Either the basolateral targeting machinery that interacts with kAE1 can tolerate the V907A substitution or kAE1(V907A) is localised to the basolateral membrane by an alternative mechanism.

The adaptor complex AP-1B, mediates basolateral sorting of some membrane proteins in epithelial cells (Folsch et al., 1999). We found that kAE1, when transiently expressed in LLC-PK1 cells (a cell line that lacks the μ1B subunit of AP1B and consequently lacks any functional AP-1B heterotetramer) had a basolateral localisation, whereas kAE1(R901Stop) had a non-polarised distribution in these cells. The C-terminal tail of kAE1 also targeted a proportion of the normally exclusively apical protein CD8 to the basolateral membrane in LLC-PK1 (Devonald et al., 2003). Taken together, these results suggest that μ1B is not necessary for kAE1 trafficking to the basolateral membrane of epithelial cells. Another adaptor complex such as AP-4 (Simmen et al., 2002) or other unidentified components might direct kAE1 to the basolateral membrane.

Chicken kAE1 has two basolateral localisation determinants in the N-terminus that are absent in human kAE1 (Adair-Kirk

et al., 1999) but the C-termini of both kAE1 orthologues are similar and contain a YxxΦ motif. Chicken kAE1 is targeted apically when the N-terminal targeting determinants are removed (Adair-Kirk et al., 1999) but much of the N-terminus homologous to human kAE1 remains present. Although the YDEV sequence in the kAE1 C-terminus can direct human kAE1 to the basolateral membrane, the YNEV sequence in the chicken C-terminus can not (Adair-Kirk et al., 1999). Our results show that the N-terminus of human kAE1, in addition to its C-terminus, is required for basolateral localisation because the protein localises to the apical surface when the N-terminal region is absent. The nature of this N-terminal basolateral determinant of human kAE1 has yet to be determined. The human kAE1 N-terminal domain might itself contain a basolateral retention signal or it might have a role in the trafficking pathway before the C-terminal tyrosine motif becomes involved. Alternatively, both the N-terminus and C-terminus might interact simultaneously with as yet unidentified targeting machinery. Inspection of the sequences of mammalian AE1 N-terminal domains showed several conserved potential tyrosine or dileucine-containing motifs that are not present in chicken AE1.

Because the mutation Y904F altered kAE1 targeting, we cannot discount the possible involvement of tyrosine phosphorylation in the basolateral localisation of kAE1. Tyrosine-based sorting signals are very similar to SH2 domain binding motifs (Songyang et al., 1993), and tyrosine phosphorylation influences the localisation of some proteins by regulating their interaction with the transport machinery (Bradshaw et al., 1997; Stephens and Banting, 1997). Both tyrosine residues, Y359 in the N-terminal domain and Y904 in the C-terminus, of AE1 are phosphorylated in red blood cells (Yannoukakos et al., 1991). Interestingly, Y359 is in a potential tyrosine-targeting motif (YKGL) that is lost in the AE1mem construct and is also absent in chicken kAE1. Further work is necessary to investigate the potential role of phosphorylation of these tyrosine residues in the polarised targeting of kAE1.

Conclusions

(1) Stably transfected human kAE1 is delivered to the basolateral membrane of polarised MDCKI cells, mimicking its localisation in the acid secreting intercalating cells of the kidney.

(2) All three dominant dRTA mutations studied cause mis-targeting of kAE1. The R589H and S613F mutations cause intracellular retention of the mutant proteins in polarised kidney cells. In these cases, the dominant disease most probably results from the mutant proteins hetero-oligomerising with the normal kAE1 and retaining the normal protein intracellularly. By contrast, the R901Stop mutation mislocalises the protein to the apical membrane of polarised cells and probably causes the disease (possibly together with mis-targeted normal protein) by preventing net acid secretion into the kidney tubular lumen.

(3) Analysis of the sequence regions required for basolateral localisation showed that Y904 in the C-terminus and determinant(s) in the N-terminal cytoplasmic domain of kAE1 are both necessary for basolateral targeting. The C-terminal PDZ-binding motif is not essential for basolateral targeting of kAE1.

(4) Unexpectedly, expression of normal kAE1 but not of kAE1(R901Stop), reduced the transepithelial electrical resistance and increased the permeability of the paracellular barrier of the polarised MDCKI monolayers.

(5) The development of this model system will allow detailed analysis of the signals and mechanisms involved in the polarised trafficking of this multispanning membrane transporter, and an understanding of the molecular mechanisms leading to distal renal tubular acidosis.

We thank N. Simmons for MDCKI cells and I. Mellman for LLC-PK1 cells. We thank D. Anstee, H. Mellor and D. Stephens for antibodies, R. Mushens for culturing antibodies and making the FITC-Bric 6 antibody, and M. Young for providing the p3.1AE1mem plasmid. We also thank the Medical Research Council for an infrastructure award to establish the School of Medical Sciences Imaging Facility and M. Jepson and A. Leard for their assistance with the imaging studies. This work was supported in part by the Wellcome Trust.

References

Adair-Kirk, T. L., Cox, K. H. and Cox, J. V. (1999). Intracellular trafficking of variant chicken kidney AE1 anion exchangers: role of alternative NH_2 termini in polarized sorting and Golgi recycling. *J. Cell Biol.* **147**, 1237-1248.

Aguilar, R. C., Boehm, M., Gorshkova, I., Crouch, R. J., Tomita, K., Saito, T., Ohno, H., and Bonifacino, J. S. (2001). Signal-binding specificity of the mu4 subunit of the adaptor protein complex AP-4. *J. Biol. Chem.* **276**, 13145-13152.

Alper, S. L., Natale, J., Gluck, S., Lodish, H. F. and Brown, D. (1989). Subtypes of intercalated cells in rat kidney collecting duct defined by antibodies against erythroid band 3 and renal vacuolar H^+-ATPase. *Proc. Natl. Acad. Sci. USA* **86**, 5429-5433.

Asai, T., Kuwahara, M., Kurihara, H., Sakai, T., Terada, Y., Marumo, F., and Sasaki, S. (2003). Pathogenesis of nephrogenic diabetes insipidus by aquaporin-2 C-terminus mutations. *Kidney Int.* **64**, 2-10.

Benting, J. H., Rietveld, A. G. and Simons, K. (1999). N-Glycans mediate the apical sorting of a GPI-anchored, raft-associated protein in Madin-Darby canine kidney cells. *J. Cell Biol.* **146**, 313-320.

Bruce, L. J., Cope, D. L., Jones, G. K., Schofield, A. E., Burley, M., Povey, S., Unwin, R. J., Wrong, O. and Tanner, M. J. A. (1997). Familial distal renal tubular acidosis is associated with mutations in the red cell anion exchanger (Band 3, AE1) gene. *J. Clin. Invest.* **100**, 1693-1707.

Bruce, L. J., Wrong, O., Toye, A. M., Young, M. T., Ogle, G., Ismail, Z., Sinha, A. K., McMaster, P., Hwaihwanje, I., Nash, G. B. et al. (2000). Band 3 mutations, renal tubular acidosis and South-East Asian ovalocytosis in Malaysia and Papua New Guinea: loss of up to 95% band 3 transport in red cells. *Biochem. J.* **350**, 41-51.

Boehm, M. and Bonifacino, J. S. (2001). Adaptins: the final recount. *Mol. Biol. Cell* **12**, 2907-2920.

Bradshaw, J. D., Lu, P., Leytze, G., Rodgers, J., Schieven, G. L., Bennett, K. L., Linsley, P. S. and Kurtz, S. E. (1997). Interaction of the cytoplasmic tail of CTLA-4 (CD152) with a clathrin-associated protein is negatively regulated by tyrosine phosphorylation. *Biochemistry* **36**, 15975-15982.

Cherqui, S., Kalatzis, V., Trugnan, G. and Antignac, C. (2001). The targeting of cystinosin to the lysosomal membrane requires a tyrosine-based signal and a novel sorting motif. *J. Biol. Chem.* **276**, 13314-13321.

Cowan, C. A., Yokoyama, N., Bianchi, L. M., Henkemeyer, M. and Fritzsch, B. (2000). EphB2 guides axons at the midline and is necessary for normal vestibular function. *Neuron* **26**, 417-430.

Dell'Angelica, E. C., Ohno, H., Ooi, C. E., Rabinovich, E., Roche, K. W. and Bonifacino, J. S. (1997). AP-3: an adaptor-like protein complex with ubiquitous expression. *EMBO J.* **16**, 917-928.

Denning, G. M., Anderson, M. P., Amara, J. F., Marshall, J., Smith, A. E. and Welsh M. J. (1992). Processing of mutant cystic fibrosis transmembrane conductance regulator is temperature-sensitive. *Nature* **358**, 761-764.

Devonald, M. A. J., Smith, A. N., Poon, J. P., Ihrke, G. and Karet, F. E. (2003). Non-polarized targeting of AE1 causes autosomal dominant distal renal tubular acidosis. *Nat. Genet.* **33**, 125-127.

Drenckhahn, D., Schluter, K., Allen, D. P. and Bennett, V. (1985). Colocalization of band 3 with ankyrin and spectrin at the basal membrane of intercalated cells in the rat kidney. *Science* **230**, 1287-1289.

Ellgaard, L. and Helenius, A. (2003). Quality control in the endoplasmic reticulum. *Nat. Rev.* **4**, 181-191.

Folsch, H., Ohno, H., Bonifacino, J. S. and Mellman, I. (1999). A novel clathrin adaptor complex mediates basolateral targeting in polarized epithelial cells. *Cell* **99**, 189-198.

Gorman, C. M. and Howard, B. H. (1983). Expression of recombinant plasmids in mammalian cells is enhanced by sodium butyrate. *Nucleic Acids Res.* **11**, 7631-7648 .

Groves, J. D., Falson, P., le Maire, M. and Tanner, M. J. (1996). Functional cell surface expression of the anion transport domain of human red cell band 3 (AE1) in the yeast Saccharomyces cerevisiae. *Proc. Natl. Acad. Sci. USA* **93**, 12245-12250.

Groves, J. D., Wang, L. and Tanner, M. J. A. (1998). Functional reassembly of the anion transport domain of human red cell band 3 (AE1) from multiple and non-complementary fragments. *FEBS Lett.* **433**, 223-227.

Gut, A., Kappeler, F., Hyka, N., Balda, M. S., Hauri, H. P. and Matter, K. (1998). Carbohydrate-mediated Golgi to cell surface transport and apical targeting of membrane proteins. *EMBO J.* **17**, 1919-1929.

Jarolim, P., Shayakul, C., Prabakaran, D., Jiang, L., Stuart-Tilley, A., Rubin, H. L., Simova, S., Zavadil, J., Herrin, J. T. et al. (1998). Autosomal dominant distal renal tubular acidosis is associated in three families with heterozygosity for the R589H mutation in the AE1 (band 3) $Cl^-/HCO3^-$ exchanger. *J. Biol. Chem.* **273**, 6380-6388.

Karet, F. E., Gainza, F. J., Gyory, A. Z., Unwin, R. J., Wrong, O., Tanner, M. J. A., Nayir, A., Alpay, H., Santos, F., Hulton, S. A. et al. (1998). Mutations in the chloride-bicarbonate exchanger gene AE1 cause autosomal dominant but not autosomal recessive distal renal tubular acidosis. *Proc. Natl. Acad. Sci. USA* **95**, 6337-6342.

Kollert-Jons, A., Wagner, S., Hubner, S., Appelhans, H. and Drenckhahn, D. (1993). Anion exchanger 1 in human kidney and oncocytoma differs from erythroid AE1 in its NH2 terminus. *Am. J. Physiol.* **265**, F813-F821.

Koivisto, U.-M., Hubbard, A. L. and Mellman, I. (2001). A novel cellular phenotype for familial hypercholesterolemia due to a defect in polarised targeting of LDL receptor. *Cell* **105**, 575-585.

Li, J., Quilty, J., Popov, M. and Reithmeier, R. A. F. (2000). Processing of N-linked oligosaccharide depends on its location in the anion exchanger, AE1, membrane glycoprotein. *Biochem. J.* **349**, 51-57.

Madrid, R., Le Maout, S., Barrault, M. B., Janvier, K., Benichou, S. and Merot, J. (2001). Polarized trafficking and surface expression of the AQP4 water channel are coordinated by serial and regulated interactions with different clathrin-adaptor complexes. *EMBO J.* **20**, 7008-7021.

Marr, N., Bichet, D. G., Lonergan, M., Arthus, M. F., Jeck, N., Seyberth, H. W., Rosenthal, W., van Os, C. H., Oksche, A. and Deen, P. M. (2002). Heteroligomerization of an Aquaporin-2 mutant with wild-type Aquaporin-2 and their misrouting to late endosomes/lysosomes explains dominant nephrogenic diabetes insipidus. *Hum. Mol. Genet.* **11**, 779-789.

Matter, K. and Mellman, I. (1994). Mechanisms of cell polarity: sorting and transport in epithelial cells. *Curr. Opin. Cell. Biol.* **6**, 545-554.

Moyer, B. D., Denton, J., Karlson, K. H., Reynolds, D., Wang, S., Mickle, J. E., Milewski, M., Cutting, G. R., Guggino, W. B., Li, M. and Stanton, B. A. (1999). A PDZ-interacting domain in CFTR is an apical membrane polarization signal. *J. Clin. Invest*. **104**, 1353-1361.

Ohno, H., Fournier, M. C., Poy, G. and Bonifacino, J. S. (1996). Structural determinants of interaction of tyrosine-based sorting signals with the adaptor medium chains. *J. Biol. Chem.* **271**, 29009-29015.

Olsen, O., Liu, H., Wade, J. B., Merot, J. and Welling, P. A. (2002). Basolateral membrane expression of the Kir 2.3 channel is coordinated by PDZ interaction with Lin-7/CASK complex. *Am. J. Physiol. Cell Physiol.* **282**, C183-C195.

Owen, D. J. and Evans, P. R. (1998). A structural explanation for the recognition of tyrosine based endocytotic signals. *Science* **282**, 1327-1332.

Quilty, J. A., Li, J. and Reithmeier, R. A. F. (2002a). Impaired trafficking of distal renal tubular acidosis mutants of the human kidney anion exchanger kAE1. *Am. J. Physiol. Renal. Physiol.* **282**, F810-F820.

Quilty, J. A., Cordat, E. and Reithmeier, R. A. F. (2002b). Impaired trafficking of human kidney anion exchanger (kAE1) caused by hetero-oligomer formation with a truncated mutant associated with distal renal tubular acidosis. *Biochem. J.* **368**, 895-903.

Richardson, J. C., Scalera, V. and Simmons, N. L. (1981). Identification of two strains of MDCK cells which resemble separate nephron tubule segments. *Biochim. Biophys. Acta.* **673**, 26-36.

Rodriguez-Boulan, E. and Gonzalez, A. (1999). Glycans in post-Golgi apical targeting: sorting signals or structural props? *Trends. Cell. Biol.* **9**, 291-294.

Schuster, V. L., Bonsib, S. M. and Jennings, M. L. (1986). Two types of collecting duct mitochondria-rich (intercalated) cells: lectin and band 3 cytochemistry. *Am. J. Physiol.* **251**, C347-C355.

Sheng, M. and Sala, C. (2001). PDZ domains and the organisation of supramolecular complexes. *Annu. Rev. Neurosci.* **24**, 1-29.

Silver, D. L. (2002). A carboxyl-terminal PDZ interacting domain of scavenger receptor B, type I is essential for cell surface expression in the liver. *J. Biol. Chem.* **277**, 34042-34047.

Simmen, T., Honing, S., Icking, A., Tikkanen, R. and Hunziker, W. (2002). AP-4 binds basolateral signals and participates in basolateral sorting in epithelial MDCK cells. *Nat. Cell. Biol.* **4**, 154-159.

Smythe, J. S., Spring, F. A., Gardner, B., Parsons, S. F., Judson, P. A. and Anstee, D. J. (1995). Monoclonal antibodies recognizing epitopes on the extracellular face and intracellular N-terminus of the human erythrocyte anion transporter (band 3) and their application to the analysis of South East Asian ovalocytes. *Blood* **85**, 2929-2936.

Stephens, D. J. and Banting, G. (1997). Insulin dependent tyrosine phosphorylation of the tyrosine internalisation motif of TGN38 creates a specific SH2 domain binding site. *FEBS Lett*. **416**, 27-29.

Stephens, D. J. and Banting, G. (1998). Specificity of interaction between adaptor-complex medium chains and the tyrosine-based sorting motifs of TGN38 and lgp120. *Biochem. J.* **335**, 567-572.

Stevenson, B. R. and Begg, D. A. (1994). Concentration-dependent effects of cytochalasin D on tight junctions and actin filaments in MDCK epithelial cells. *J. Cell Biol*. **107**, 367-375.

Songyang, Z., Shoelson, S. E., Chaudhuri, M., Gish, G., Pawson, T., Haser, W. G., King, F., Roberts, T., Ratnofsky, S., Lechleider, R. J. et al. (1993). SH2 domains recognize specific phosphopeptide sequences. *Cell* **72**, 767-778.

Sun, A. Q., Arrese, M. A., Zeng, L., Swaby, I., Zhou, M. M. and Suchy, F. J. (2001). The rat liver Na(+)/bile acid cotransporter. Importance of the cytoplasmic tail to function and plasma membrane targeting. *J. Biol. Chem*. **276**, 6825-6833.

Tanner, M. J. A. (2002). Band 3 anion exchanger and its involvement in erythrocyte and kidney disorders. *Curr. Opin. Hematol.* **9**, 133-139.

Takakuwa, R., Kokai, Y., Kojima, T., Akatsuka, T., Tobioka, H., Sawada, N. and Mori, M. (2000). Uncoupling of gate and fence functions of MDCK cells by the actin depolymerising reagent Mycalolide B. *Exp. Cell Res.* **257**, 238-244.

Toye, A. M., Bruce, L. J., Unwin, R. J., Wrong, O. and Tanner, M. J. A. (2002). Band 3 Walton, a C-terminal deletion associated with distal renal tubular acidosis, is expressed in the red cell membrane but retained internally in kidney cells. *Blood* **99**, 342-347.

Wainwright, S. D., Tanner, M. J. A., Martin, G. E., Yendle, J. E. and Holmes, C. (1989). Monoclonal antibodies to the membrane domain of the human erythrocyte anion transport protein. *Biochem. J.* **258**, 211-220.

Wilde, A., Reaves, B. and Banting, G. (1992). Epitope mapping of two isoforms of a trans Golgi network specific integral membrane protein TGN38/41. *FEBS Lett.* **13**, 235-238.

Yannoukakos, D., Meyer, H. E., Vasseur, C., Driancourt, C., Wajcman, H. and Bursaux, E. (1991). Three regions of erythrocyte band 3 protein are phosphorylated on tyrosines: characterization of the phosphorylation sites by solid phase sequencing combined with capillary electrophoresis. *Biochim. Biophys. Acta.* **1066**, 70-76.