Biochem. J. (2002) **368**, 895–903 (Printed in Great Britain)

Impaired trafficking of human kidney anion exchanger (kAE1) caused by hetero-oligomer formation with a truncated mutant associated with distal renal tubular acidosis

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Autosomal dominant distal renal tubular acidosis (dRTA) has been associated with several mutations in the anion exchanger AE1 gene. The effect of an 11-amino-acid C-terminal dRTA truncation mutation (901 stop) on the expression of kidney AE1 (kAE1) and erythroid AE1 was examined in transiently transfected HEK-293 cells. Unlike the wild-type proteins, kAE1 901 stop and AE1 901 stop mutants exhibited impaired trafficking from the endoplasmic reticulum to the plasma membrane as determined by immunolocalization, cell-surface biotinylation, oligosaccharide processing and pulse–chase experiments. The 901 stop mutants were able to bind to an inhibitor affinity resin, suggesting that these mutant membrane proteins were not grossly misfolded. Co-expression of wild-type and mutant kAE1 or AE1 resulted in intracellular retention of the wild-type proteins in a pre-medial Golgi compartment. This dominant negative effect was due to hetero-oligomer formation of the mutant and wild-type proteins. Intracellular retention of kAE1 in the α-intercalated cells of the kidney would account for the impaired acid secretion into the urine characteristic of dRTA.

Key words: band 3, biosynthesis, membrane protein.

INTRODUCTION

Human anion exchanger 1 (AE1, or band 3) is an abundant 911-amino-acid membrane glycoprotein localized in the plasma membrane of red cells [1–3]. The N-terminal cytoplasmic domain binds to cytoskeletal proteins, ankyrin, band 4.1 and band 4.2, as well as glycolytic enzymes and haemoglobin [4]. The C-terminal membrane domain of AE1 has been proposed to span the membrane 12 times [5] and catalyses the electro-neutral exchange of chloride for bicarbonate [6]. AE1 exists as a mixture of dimers and tetramers in the red cell membrane [7].

A kidney form of AE1 (kAE1) is located in the basolateral membrane of acid-secreting (α) intercalated cells of the collecting duct of the kidney [8–12]. Due to an alternative kidney promoter, human kAE1 has a truncated N-terminus, beginning at Met-66 [11,13]. In the α-intercalated cells, carbon dioxide is hydrated by carbonic anhydrase II to form a proton and bicarbonate. The protons are pumped into the urine by apical H^+-ATPases, while kAE1 provides a pathway for bicarbonate reabsorption across the basolateral membrane into the blood.

Distal renal tubular acidosis (dRTA) is a disease characterized by impaired proton secretion into the urine, leading to metabolic acidosis, hypokalaemia, bone disease and nephrocalcinosis [14,15]. Several mutations in the AE1 gene (SLC4A1) were found to be associated with autosomal dominant dRTA [16–20] and two with autosomal recessive dRTA [21,22]. A current hypothesis is that the dRTA mutations cause mistargeting of kAE1 in epithelial cells, impairing bicarbonate movement across the basolateral membrane [23]. Missense mutants (R589H, R589C and S613F) associated with autosomal dominant dRTA were functional when either the erythroid AE1 or kAE1 forms were expressed in *Xenopus* oocytes [19,23]. Interestingly, the R589H mutation caused intracellular retention of kAE1, but not AE1, in transfected kidney-derived HEK-293 cells [24]. This would account for the impaired kidney but normal red-cell anion-transport function.

The AE1 G701D mutation, linked to autosomal recessive dRTA, caused impaired trafficking of mutant erythroid AE1 and kAE1 in *Xenopus* oocytes [21]. Functional cell-surface expression of the mutants could be rescued by co-expression of the red cell protein glycophorin A. It was hypothesized that kAE1 G701D had impaired trafficking to the basolateral membrane in kidney cells, while erythroid AE1 G701D could traffic to the red cell plasma membrane due to the presence of glycophorin A in the red cell precursors.

Two brothers diagnosed with autosomal dominant dRTA have a 13 bp duplication from codon 896 to codon 900 in one of their AE1 genes, resulting in the creation of a premature stop codon at position 901 and deletion of the C-terminal 11 residues [18]. A recent report [25] found intracellular retention of the kAE1 mutant in transfected Madin–Darby canine kidney (MDCK) cells. In this paper, we examined the effect of this 11-amino acid deletion (901 stop) on the biosynthesis, folding and trafficking of AE1 and kAE1 in transfected HEK-293 cells. The 901 stop mutants in both the erythroid and kidney forms showed impaired trafficking to the plasma membrane. Co-expression of the wild-type and mutant proteins, mimicking the heterozygous state of the patients, resulted in hetero-oligomer formation and

Abbreviations used: AE1, anion exchanger 1; kAE1, kidney AE1; $C_{12}E_8$, octaethylene glycol mono-*n*-dodecyl ether; dRTA, distal renal tubular acidosis; endo H, endoglycosidase H; ER, endoplasmic reticulum; H_2DIDS, 4,4′-di-isothiocyanato-2,2′-dihydrostilbenedisulphonate; MDCK, Madin–Darby canine kidney; PNGase F, peptide N-glycosidase F; SAO, Southeast Asian ovalocytosis; SITS, 4-acetamido-4′-isothiocyanostilbene-2,2′-disulphonate.

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impaired trafficking of the wild-type protein to the medial Golgi. The altered trafficking of kAE1 901 stop and the dominant negative effect provide an explanation for the lack of proper urine acidification and the dominant nature of this dRTA mutation.

MATERIALS AND METHODS

Construction of plasmid and mutations

Human AE1 cDNA (a generous gift of Dr A. M. Garcia and Dr H. Lodish, Whitehead Institute, Cambridge, MA, U.S.A.) was inserted into the *Hin*dIII and *Bam*HI sites of pcDNA3 (Invitrogen, Carlsbad, CA, U.S.A.). The 901 stop mutations were created by mutating the 901 codon to a stop codon using the QuikChange™ site-directed mutagenesis kit from Stratagene (La Jolla, CA, U.S.A.) and oligonucleotide primers from ACGT Corp. (Toronto, Canada). The kidney form (kAE1) [24], the N555 mutants [26], the His_6-tagged AE1 [24] and the Southeast Asian ovalocytosis (SAO) mutant [5] were constructed as described previously. The kidney form of AE1-His6 was created by digestion and ligation of the AE1-His6 as described for the creation of kAE1 [24]. The mutations were confirmed using a T7 Sequencing™ Kit (Amersham Biosciences, Piscataway, NJ, U.S.A.) and [^{35}S]dATP (NEN Life Sciences Products, Boston, MA, U.S.A.). Plasmid DNA for transfections was purified with Qiagen (Valencia, CA, U.S.A.) Plasmid Midi columns.

Cell culture

HEK-293 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10 % calf serum and 0.5 % penicillin and streptomycin (Gibco Life Technologies, Gaithersburg, MD, U.S.A.) in 5 % CO_2 at 37 °C. The day before transfection the cells were collected by trypsin (0.05 % w/v) digest, and replated in six-well plates. The DEAE-dextran method [27] was used to transiently transfect the cells (0.8 μg of DNA/well of a six-well dish). As described below, the cell extracts were prepared by detergent solubilization 24–48 h after transfection.

Immunochemistry

Transiently transfected HEK-293 cells were fixed for 20 min in 3.8 % formaldehyde before washing once with 100 mM glycine in PBS, pH 7.4. Cells were then permeabilized for 15 min in PBS containing 0.2 % Triton X-100, washed three times in PBS and incubated in blocking buffer (PBS/3 % BSA) for 1 h at room temperature. Anti-kAE1 N-terminal rabbit antibody in blocking buffer was then added to the cells for 30 min at room temperature before three washes in PBS. Cells were then incubated with Cy3-conjugated anti-rabbit antibody (Jackson Immunoresearch Laboratories, West Grove, PA, U.S.A.) diluted 1/500 for 30 min in blocking buffer. After three washes with PBS, coverslips were mounted on to slides before observation using a laser scanning confocal Zeiss LSM 510 microscope.

Cell-surface biotinylation

Cell-surface proteins were biotinylated as described previously [28]. Briefly, 48 h after transfection, HEK-293 cells were washed with cold borate buffer (10 mM boric acid, 154 mM NaCl, 7.2 mM KCl and 1.8 mM $CaCl_2$, pH 9.0). Cells were treated twice with 0.8 mM EZ-Link NHS-SS-Biotin (Pierce, Rockford, IL, U.S.A.) in borate buffer for 30 min at room temperature. The cells were then rinsed with 0.192 M glycine/25 mM Tris, pH 8.3, solution to quench and remove any unreacted reagent. The cells were lysed in RIPA buffer (1 % deoxycholic acid, 1 % Triton X-100, 0.1 % SDS, 0.15 M NaCl, 1 mM EDTA and 10 mM Tris/HCl, pH 7.5) containing protease inhibitors: 200 μM PMSF (Sigma-Aldrich, St. Louis, MO, U.S.A.), 2.8 μM E64 (Sigma-Aldrich), 1 μM leupeptin (Boehringer Mannheim) and 1 μM pepstatin (Boehringer Mannheim). An aliquot of the lysate (total) was saved for Western blotting. ImmunoPure (Pierce)-immobilized streptavidin was added to bind the biotinylated proteins. The supernatant (unbound) was removed and an aliquot was saved for Western blotting. After washing the streptavidin beads, SDS sample buffer (containing 5 % β-mercaptoethanol) was added to the beads and the samples (bound) were incubated for 1 h at room temperature to cleave the disulphide bond in the biotinylating reagent and to release the captured proteins. Samples were analysed for AE1 content by Western blotting.

Western blotting

SDS/PAGE and Western blotting of cell lysate samples were performed as described previously [28]. AE1 and AE1 901 stop proteins were detected using rabbit antiserum (anti-AE1-Nt) raised against a synthetic peptide of the first 16 amino acids of AE1, including an acetylated N-terminus. This antibody did not detect kAE1, which is missing the first 65 residues of AE1. kAE1 and kAE1 901 stop proteins were detected using a rabbit antiserum (anti-kAE1-Nt) raised against synthetic peptides of the first 16 amino acids of kAE1, including an acetylated N-terminus. This antibody did not detect AE1, although it contained the peptide sequence. This suggests that the antibody binds preferentially to the free N-terminus of kAE1. AE1, kAE1 and SAO AE1 could be detected using an antibody serum (anti-AE1-Ct) raised in rabbit against a peptide of the last 16 amino acids of human AE1 [5]. This antibody cannot detect the C-terminal truncation mutants or the C-terminal His-tagged version (AE1-His6).

Enzymic deglycosylation

Transfected cells were lysed in PBS containing 1 % octaethylene glycol mono-*n*-dodecyl ether ($C_{12}E_8$; Nikko, Tokyo, Japan) and protease inhibitors (PMSF, leupeptin, pepstatin A and E64) at 4 °C. The lysates were centrifuged (16000 ***g***) at 4 °C to remove insoluble material. Aliquots (40 μl) of cell lysate were treated with 1000 units of endoglycosidase H (endo H; New England Biolabs) or 500 units of peptide N-glycosidase F (PNGase F; New England Biolabs) at room temperature for 1 h, followed by the addition of 1 vol. of 2 × Laemmli sample buffer containing 4 % (w/v) SDS. AE1 and kAE1 were detected by Western blotting.

Pulse–chase assay

One day after transfection, the cells were pulsed for 20 min with 200 μCi/ml L-[^{35}S]methionine (NEN Life Science Products) in methionine-free Dulbecco's modified Eagle's medium (Gibco Life Technologies) [28]. After labelling, the medium was removed and replaced with Dulbecco's modified Eagle's medium. Each chase time point was a single well of a six-well plate. At various time points the cells were lysed in RIPA buffer containing protease inhibitors (PMSF, leupeptin, pepstatin A and E64). AE1 and kAE1 were immunoprecipitated with antibodies against the AE1 and kAE1 N-termini, respectively, followed by addition of Protein G–Sepharose (Amersham Biosciences). The immunoprecipitates were analysed by electrophoresis using SDS/PAGE

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(8 % gels). The gels were dried and exposed to film and a Phosphoimager screen to visualize the radiolabelled AE1.

SITS (4-acetamido-4′-isothiocyanostilbene-2,2′-disulphonate)–Affi Gel binding assay

Transfected cells were lysed in PBS containing 1 % $C_{12}E_8$ and protease inhibitors (PMSF, leupeptin, pepstatin A and E64) at 4 °C. The lysates were centrifuged (16000 ***g***) to remove insoluble material. Immunoblotting showed that all of the AE1 was solubilized and that the pellet was devoid of AE1. SITS was conjugated to Affi-Gel 102 (Bio-Rad, Hercules, CA, U.S.A.), as described in [29]. SITS-Affi Gel (25 μl) and 100 μl of lysate were added to 875 μl of binding buffer (1 % $C_{12}E_8$ and 228 mM sodium citrate buffer, pH 7.1, final concentration) with or without free anion-transport inhibitor, 4,4′-di-isothiocyanato-2,2′-dihydrostilbenedisulphonate (H_2DIDS; 1 mM; Sigma-Aldrich). The mixture was incubated at 4 °C for 15 min. Resin was collected by centrifugation (8000 ***g***) for 5 s and washed three times with 0.1 % $C_{12}E_8$/228 mM sodium citrate buffer, pH 7.1. SDS sample buffer (4 % SDS) was added to the SITS-Affi Gel to elute bound proteins and the total and bound fractions were analysed for AE1 content by Western blotting.

Co-expression and purification of His-tagged AE1

Cells were transfected with N555 cDNA alone, N555 cDNA with increasing amounts of 901 stop mutant cDNA, or 901 stop cDNA alone. This was performed for both erythroid AE1 and kAE1. Transfected cells were lysed in PBS, pH 7.4, containing 1 % $C_{12}E_8$ and protease inhibitors at 4 °C. AE1 and kAE1 in the lysate were detected by Western blotting. To detect hetero-oligomers of wild-type and mutant AE1 proteins, a His-tagged version of AE1 was constructed. This protein and any associated proteins can be purified from transfected cells by Ni^{2+}-affinity chromatography [24]. Cells were either transfected with AE1 or AE1-His6 alone, or co-transfected with AE1-His6 in combination with AE1, AE1 901 stop, kAE1 or kAE1 901 stop. Other cells were transfected with kAE1-His6 alone or co-transfected with kAE1-His6 and kAE1 901 stop. Transfected cells were lysed in PBS, pH 7.4, containing 1 % $C_{12}E_8$, 5 mM imidazole and protease inhibitors at 4 °C. A portion (300 μl) of lysate was incubated with 40 μl of ProBond™ nickel beads (Invitrogen) for 1 h at 4 °C. Beads were washed three times with 0.5 ml of PBS, pH 7.4/0.1 % $C_{12}E_8$/40 mM imidazole. Bound proteins were eluted with the same buffer containing 500 mM imidazole. AE1 in the eluate was detected by Western blotting with anti-AE1-Ct antibody to detect full-length protein, and anti-AE1-Nt and anti-kAE1-Nt antibodies to detect truncated proteins and His_6-tagged proteins.

RESULTS

Localization of kAE1 in transfected HEK-293 cells

Immunofluorescence staining was used to determine the subcellular location of kAE1 and kAE1 901 stop in transfected HEK-293 cells. The anti-N-terminus antibody readily detected kAE1 in fixed, permeabilized cells (Figure 1). Cells transfected with wild-type kAE1 showed intense immunofluorescence at the cell periphery (Figure 1A), consistent with a high level of cell-surface expression as previously determined [24]. In contrast, cells transfected with kAE1 901 stop showed predominantly intracellular staining (Figure 1B), without the intense cell-surface staining seen in wild-type kAE1 transfected cells. AE1 901 stop also showed predominantly internal staining (results not shown).

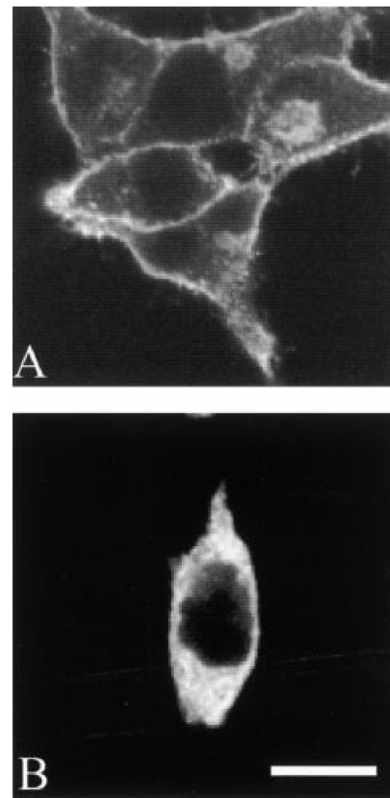

Figure 1 Immunolocalization of kAE1 and kAE1 901 stop in transfected HEK-293 cells

HEK-293 cells expressing kAE1 (**A**) or kAE1 901 stop (**B**) were fixed for 20 min in 3.8% formaldehyde, permeabilized in 0.2% Triton X-100 and blocked for 1 h using 3% BSA in PBS. Cells were first incubated with anti-kAE1-Nt rabbit antibody and then with Cy3-conjugated anti-rabbit antibody (1/500) to visualize the proteins. Scale bar, 10 μm.

In control experiments, mock-transfected cells (not shown) showed background immunofluorescence staining. The immunofluorescence images suggest that while kAE1 was expressed at the cell surface, kAE1 901 stop was predominantly localized intracellularly and had greatly reduced cell-surface expression.

901 stop mutants are not detectable at the cell surface

To confirm the immunolocalization data, cell-surface biotinylation was used to determine the amount of plasma-membrane expression of kAE1 and AE1 in transfected HEK-293 cells (Figure 2). The levels of total expression of kAE1, AE1 and the 901 stop mutants, as determined by immunoblotting of cell extracts (Figure 2, lanes T), were very similar. This suggests that the mutants were not grossly misfolded and targeted for rapid degradation. Wild-type kAE1 and AE1 could be detected in the biotinylated fraction (Figure 2, lanes B). In contrast, no kAE1 901 stop or AE1 901 stop was detectable in the bound fraction. This suggests that the mutant proteins were poorly expressed at the cell surface relative to the wild-type proteins.

The amount of kAE1 or AE1 in the biotinylated fraction (Figure 2, lanes B) relative to the total expression (Figure 2, lanes T) should give the value for cell-surface expression. However, biotinylated kAE1 and AE1 are eluted very poorly from streptavidin beads, as noted previously [26,28,30]. The percentage of cell-surface expression was therefore determined by comparing the pixel density of unbiotinylated kAE1 or AE1 in the supernatant

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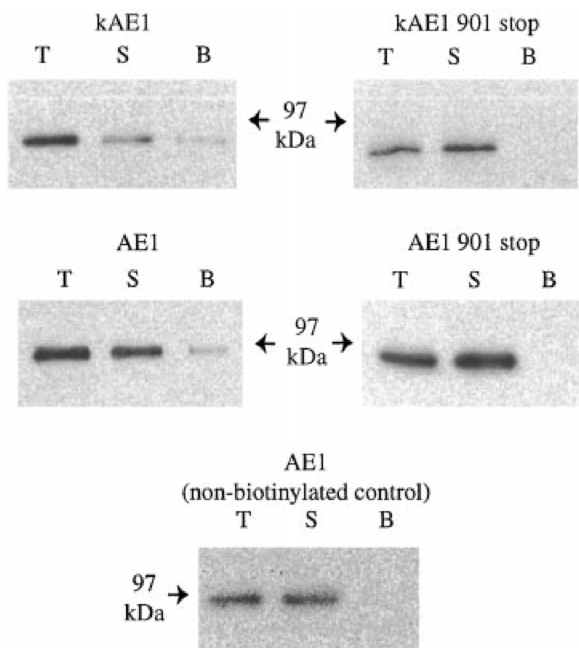

Figure 2 Expression and cell-surface biotinylation of transfected HEK-293 cells

The immunoblots show the amount of total AE1 in the cell extract (lanes T), the amount of AE1 not bound to streptavidin beads (lanes S) and the amount of AE1 eluted from the streptavidin beads (lanes B). The fraction bound to the beads was 5 × overloaded with respect to the total and supernatant to get comparable band densities within the linear range of the chemiluminescence exposure. AE1 and AE1 901 stop were detected with the anti-AE1-Nt antibody, and kAE1 and kAE1 901 stop were detected with the anti-kAE1-Nt antibody. The position of a 97 kDa molecular-mass marker is indicated by an arrow.

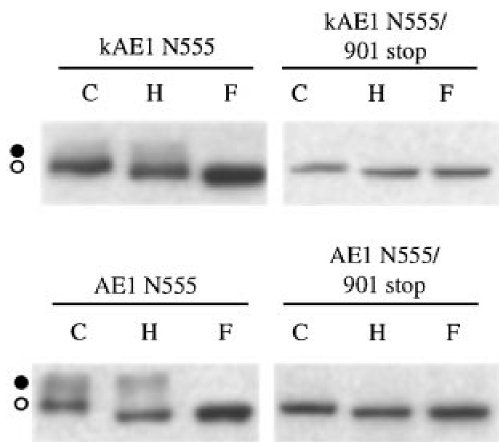

Figure 3 Enzymic deglycosylation of N555 constructs

Transfected cells were lysed in 1% $C_{12}E_8$ and extracts were incubated with no enzyme (lanes C), endo H (lanes H) or PNGase F (lanes F). Western blotting was used to detect AE1. ●, the complex form; ○, the high-mannose form. AE1 and AE1 901 stop were detected with the anti-AE1-Nt antibody and kAE1 and kAE1 901 stop were detected with the anti-kAE1-Nt antibody.

after incubation with streptavidin beads (Figure 2, lanes S) with the total expression of kAE1 or AE1 (Figure 2, lanes T). For kAE1 and AE1, a reduction in the amount of protein in the unbound fraction relative to the total was clearly evident, while no reduction was observed for the 901 stop mutants or the non-biotinylated control (Figure 2, compare lanes S and T). Using the difference in AE1 density in the supernatant fraction and total fraction, the percentage cell-surface expression was determined to be as follows: kAE1, 48±9%; kAE1 901 stop, −6±7%; AE1, 40±8%; AE1 901 stop, 4±13% ($n = 3$; ±S.D.). This level of cell-surface expression of kAE1 and AE1 is similar to previous reports [24,26,28,30]. This indicates that the wild-type kAE1 and AE1 were expressed at the cell surface at similar levels, while the mutant proteins had little or no cell-surface expression.

Cells transfected with AE1 but not treated with the biotinylating reagent only showed a small decrease (4±9%, $n = 3$) in AE1 content in the supernatant compared with the total fraction, indicating that there was only a low level of non-specific binding of AE1 to the streptavidin beads. Also, no non-biotinylated AE1 control was detectable in the biotinylated fraction (Figure 2, lanes B), again suggesting a lack of non-specific binding.

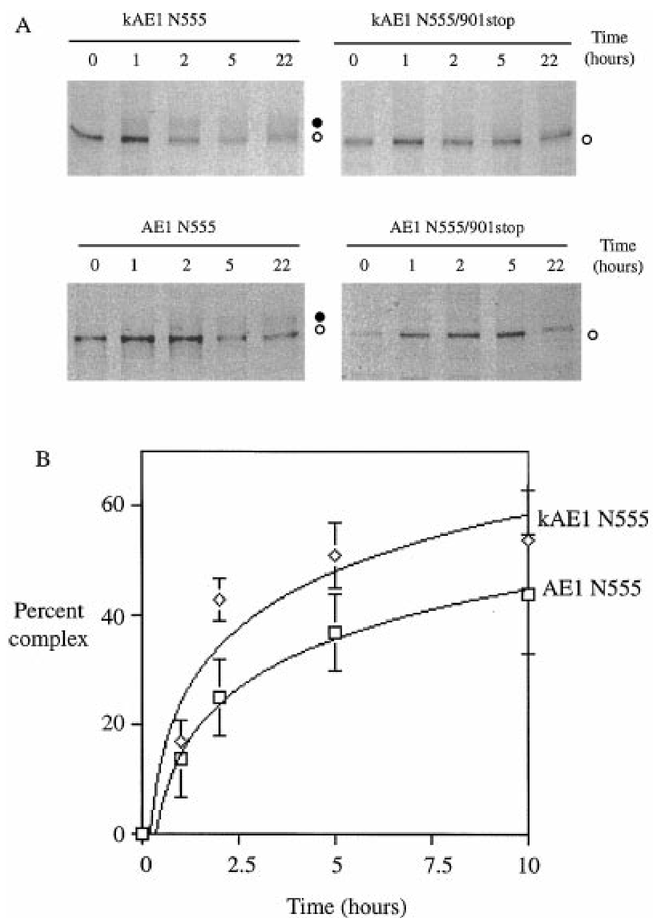

Figure 4 Pulse–chase analysis of N555 constructs

(**A**) Transfected HEK-293 cells were pulsed with 200 μCi/ml [^{35}S]methionine for 20 min. Cells were collected at 0, 1, 2, 5 and 22 h of the chase, and AE1 and kAE1 were immunoprecipitated using the anti-AE1-Nt antibody and the anti-kAE1-Nt antibody, respectively. Samples were treated with endo H to improve the separation of complex and high-mannose forms. Radiolabelled AE1 and kAE1 were visualized by SDS/PAGE and autoradiography. ●, the complex form; ○, the high-mannose form. (**B**) The percentage of complex form of kAE1 N555 and AE1 N555 are shown as a function of time. ◇, kAE1 N555; □, AE1 N555. Data are means ± S.D. from three separate pulse–chase experiments.

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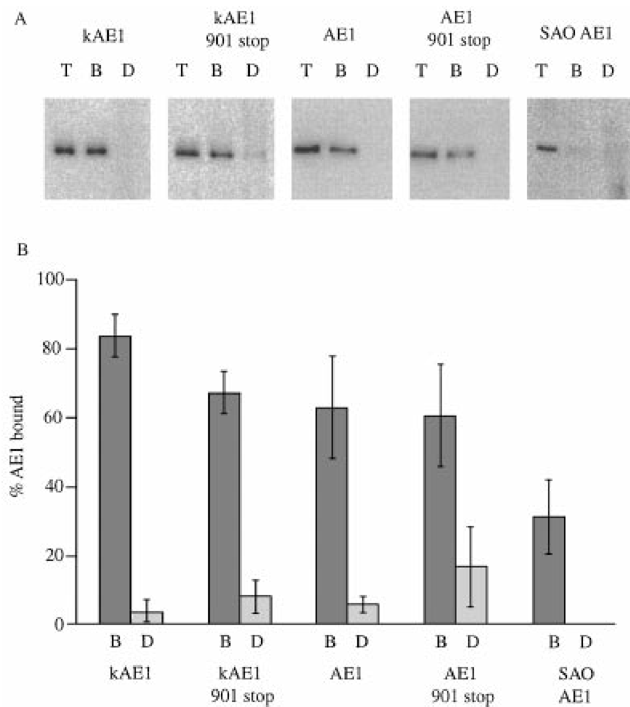

Figure 5 SITS-Affi Gel binding of wild-type and mutant kAE1

(**A**) The immunoblot shows the total kAE1, kAE1 901 stop and AE1 SAO in extracts prepared from transfected cells (lanes T), the amount bound to the SITS-Affi Gel resin in the absence (lanes B) or presence (lanes D) of 1 mM H_2DIDS. AE1 and AE1 901 stop were detected with the anti-AE1-Nt antibody and kAE1 and kAE1 901 stop were detected with the anti-kAE1-Nt antibody. (**B**) The histogram shows the relative amounts of kAE1, AE1 and AE1 SAO in extracts prepared from transfected HEK-293 cells that bound to the SITS-Affi Gel resin in the absence (dark bars) or presence (light bars) of 1 mM H_2DIDS. Error bars represent S.D. ($n = 3$).

Altered glycosylation of 901 stop mutants

As glycoproteins move through the secretory pathway, their oligosaccharides are usually processed from high mannose to a complex form. Glycosidases can be used to determine the type of oligosaccharides present on glycoproteins. Endo H cleaves high-mannose oligosaccharides, while PNGase F cleaves all N-linked oligosaccharides from glycoproteins. Glycoproteins that reach the medial Golgi can be converted into an endo H-insensitive form by the activity of α1,3/6-mannosidase II [31]. As determined by endo H-sensitivity, AE1 and kAE1 in transfected HEK-293 cells contain only high-mannose oligosaccharide at the endogenous glycosylation acceptor site, N642, even though both proteins traffic to the plasma membrane [5,24,26]. Previous studies [24,26] have shown that moving the oligosaccharide acceptor site to position 555 (AE1 N555 and kAE1 N555) on the preceding extracellular loop now enables the oligosaccharide to be processed from an endo H-sensitive high-mannose structure to an endo H-insensitive complex structure. The complex form of AE1 N555 was shown to be at the plasma membrane [26]. Processing of the oligosaccharide on N555 mutants from a high-mannose structure to a complex structure indicates that the protein has reached the medial Golgi.

Western blotting of lysates of HEK-293 cells transfected with kAE1 N555 or AE1 N555 showed two immunoreactive bands (Figure 3, lanes C). The lower band was sensitive to endo H (Figure 3, lanes H), seen by its increased mobility after removal of the oligosaccharide, indicating that it contained high-mannose oligosaccharide. The upper band did not have altered mobility, indicating that it did not contain high-mannose oligosaccharide. Both bands had increased mobility after treatment with PNGase F, indicating that they both contained N-linked oligosaccharides. The upper band contains complex oligosaccharide corresponding to protein that has trafficked to the medial Golgi. The lower band contains high-mannose oligosaccharide that corresponds to protein located in a pre-medial Golgi compartment [24,26]. Approx. 30–35 % of total kAE1 N555 and AE1 N555 contained complex oligosaccharide, agreeing with the cell-surface biotinylation results and previous studies [24,26] that a considerable portion of kAE1 and AE1 is present at the plasma membrane in transfected HEK-293 cells.

In contrast, lysates of cells transfected with AE1 N555/901 stop or kAE1 N555/901 stop showed only a single immunoreactive band that was sensitive to endo H (Figure 3). The absence of a complex form of the truncated mutants suggests that the proteins had not reached the medial Golgi and were impaired in their trafficking to the plasma membrane.

N-linked oligosaccharides on 901 stop mutants are not processed to complex form

The rate of trafficking of AE1 and kAE1 from the endoplasmic reticulum (ER) to the medial Golgi was monitored by observing the conversion of the N555 constructs from a high-mannose into a complex form in a pulse–chase experiment. At the initial time point of the chase (Figure 4A, time 0), the [^{35}S]methionine-labelled kAE1 N555 and AE1 N555 contained high-mannose oligosaccharides, as indicated by a single lower band (Figure 4A). Over the duration of the chase, an endo H-insensitive band with slower mobility appeared, as previously observed [24,26]. This represents the processing of the high-mannose oligosaccharide to a complex structure, as the protein moves from the ER to the medial Golgi. After three separate pulse–chase experiments, the percentage of complex AE1 N555 and kAE1 N555 at each time point was quantified by densitometry (Figure 4B). The complex form of kAE1 N555 appeared sooner than AE1 N555. For example, after 2 h of chase the amounts of complex kAE1 N555 and AE1 N555 were approx. 40 and 20 %, respectively. This suggests that kAE1 N555 exits the ER more rapidly. The percentages of complex kAE1 N555 and AE1 N555 were more similar at later chase time points.

At the initial time point, the AE1 N555/901 stop and kAE1 N555/901 stop mutants also showed a single band. However, over the time of the chase there was no appearance of an upper endo H-insensitive band, indicating that very little of the oligosaccharide on the mutant proteins was processed to a complex structure. This result suggests that the truncation mutants are retained in a pre-medial Golgi compartment, probably the ER.

Often, mutant membrane proteins that are retained within the ER are also degraded rapidly [32]. The pulse–chase experiments show that there was a comparable amount of all four proteins at 22 h. This suggests that the mutant proteins were not targeted for more rapid degradation than the wild-type protein, in agreement with the immunoblotting results.

901 stop mutants bind to an inhibitor affinity resin

Intracellular retention of mutant membrane proteins is often caused by their incomplete folding or misfolding [32,33]. The ability of the mutant kAE1 and AE1 to bind to an inhibitor affinity resin was used to provide a measure of proper folding of

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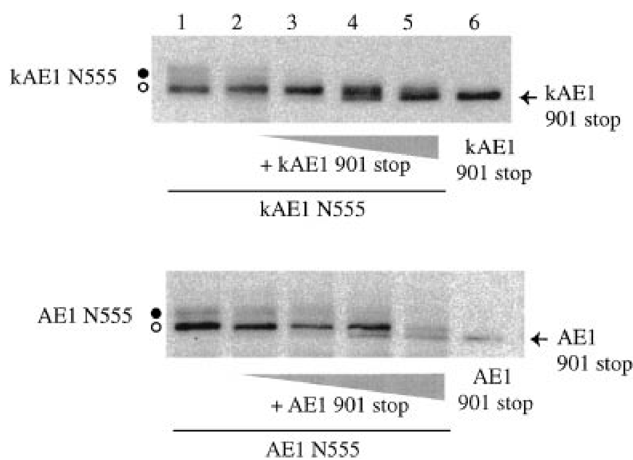

Figure 6 Co-expression of N555 and 901 stop AE1 and kAE1

Transfected cells were lysed with 1% $C_{12}E_8$, and AE1 and kAE1 in the cell extracts were detected by Western blotting. The top panel shows results from co-expression of AE1 N555 and AE1 901 stop, while the bottom panel shows results from co-expression of kAE1 N555 and kAE1 901 stop. Cells were transfected with N555 alone (0.8 μg cDNA/well, lanes 1), N555 (0.8 μg cDNA/well) with increasing amounts of 901 stop (0.08, 0.2, 0.4 or 0.8 μg cDNA/well, lanes 2–5 respectively) or 901 stop alone (0.8 μg cDNA/well, lanes 6). ●, Complex form; ○, high-mannose forms. AE1 and AE1 901 stop were detected with the anti-AE1-Nt antibody and kAE1 and kAE1 901 stop were detected with the anti-kAE1-Nt antibody. The arrows indicate the position of the 901 stop mutant.

the inhibitor-binding site of AE1 expressed in transfected cells, including the fraction localized in intracellular compartments. The SITS-Affi Gel has been shown to specifically bind to properly folded AE1 isolated from red blood cells [29] and transfected cells [28]. Over 80% of wild-type kAE1 was able to bind to the SITS-Affi resin under the conditions used (Figure 5, lanes B relative to lanes T). The binding was almost completely blocked with unconjugated H_2DIDS (Figure 5, lanes D), indicating that the binding was specific. The kAE1 901 stop mutant also bound to the SITS-Affi resin (70% binding) and the binding was also blocked by free H_2DIDS. The AE1 901 stop mutant also had similar binding to the SITS-Affi resin as wild-type AE1 (Figure 5). The SAO AE1 mutant contains a nine-amino acid deletion from Ala-400 to Ala-408. SAO AE1 is misfolded and has been shown previously to be unable to transport anions or to bind stilbene disulphonates [34–36]. The binding of SAO AE1 to the SITS-Affi resin (only 30% binding) was determined to be significantly less than kAE1, AE1 and the 901 stop mutants. The results show that the 901 stop mutants were able to bind anion-exchange inhibitors similar to the wild-type proteins and were therefore not grossly misfolded.

901 stop mutants cause a dominant negative effect

Individuals with the dRTA 901 stop mutation in the AE1 gene are heterozygotes with a normal and a mutant AE1 allele [18]. Their α-intercalated kidney cells would be expected to express both the wild-type and mutant proteins. The effect of the mutant proteins on trafficking of normal AE1 and kAE1 was examined by co-expression of normal and mutant AE1 or kAE1. AE1 N555 and kAE1 N555 were used as normal samples so that their trafficking to the Golgi could be monitored by the amount of complex form. When AE1 N555 or kAE1 N555 were expressed alone, a lower high-mannose band and an upper complex band were observed as before (Figure 6, lanes 1). As increasing amounts of mutant cDNA (AE1 901 stop or kAE1 901 stop) were co-transfected (Figure 6, lanes 2–5), another band emerged that co-migrated with the 901 stop mutants (Figure 6, lanes 6), indicating increasing amounts of co-expressed mutant protein. As the amount of mutant protein expression increased, there was a decrease in the amount of AE1 N555 and kAE1 N555 complex forms, indicating reduced trafficking of the wild-type proteins to the medial Golgi. This suggests that the 901 stop mutants caused a dominant negative effect on the trafficking of wild-type proteins.

901 stop mutants co-purify with His-tagged AE1 and kAE1

Since AE1 exists mainly as a dimer in erythrocytes [7] and transfected HEK-293 cells [28,37], the dominant negative effect may by caused by heterodimerization of wild-type and mutant proteins. Intracellular retention of the 901 stop mutant would then lead to retention of the associated wild-type AE1. Co-purification of wild-type and mutant AE1 from co-transfected cells was performed to determine whether heterodimers could form. C-terminal His_6-tagged AE1 and a His_6-tagged kAE1 were used so that the purification could be accomplished using Ni^{2+}-affinity chromatography. The N555 form of the His-tagged AE1 had an approx. 30% complex form on Western blots (results not shown), similar to AE1 N555, suggesting that the His_6 tag did not greatly alter the trafficking of AE1 from the ER to the medial Golgi.

Lysates of transfected cells were incubated with Ni^{2+} beads. AE1 or kAE1 without a His tag do not bind the Ni^{2+} beads [24]. AE1-His and any associated proteins were eluted with imidazole, and AE1 and kAE1 in the eluted fractions were detected by immunoblotting (Figure 7). For convenience, AE1-His and the C-terminally truncated AE1 and kAE1 were detected with a mixture of N-terminal antibodies (Figure 7A). AE1-His is not detected by the C-terminal antibody, since the C-terminal His tag disrupts the antibody epitope [24]. The 901 stop truncation mutants are also not detected by the C-terminal antibody, since they do not contain the epitope. The C-terminal antibody detects only the full-length AE1 or kAE1.

AE1 His was expressed alone or in combination with AE1 or kAE1 in HEK-293 cells. When AE1-His was transfected alone (Figure 7, lane 1), AE1-His could be detected in the eluted fraction by the N-terminal antibodies (Figure 7A), indicating that AE1-His bound to and could be eluted from the Ni^{2+} beads with imidazole. When co-transfected with AE1-His, both AE1 and kAE1 co-purified with AE1-His (Figure 7, lanes 2 and 4, respectively), as described previously [24]. AE1 and AE1-His cannot be readily resolved on SDS gels (Figure 7A, lane 2). Using the C-terminal antibody, which does not recognize AE1-His, normal AE1 was shown to co-purify with AE1-His (Figure 7B, lane 2). Wild-type kAE1 could be detected by the anti-N-terminal and the anti-C-terminal antibodies in the eluted fraction (Figures 7A and 7B, lanes 4), showing that it co-purified with AE1-His.

AE1-His was then co-transfected with kAE1 901 stop or AE1 901 stop. AE1-His and the 901 stop mutants were detected in the eluant from the Ni^{2+} beads (Figure 7A, lanes 3 and 5). The 901 stop mutants could be resolved on SDS gels from AE1-His due to their smaller size, and were detected by blotting with the N-terminal antibodies. kAE1 901 stop and AE1 901 stop co-purified with AE1-His from lysate of co-transfected cells.

To examine the interaction between wild-type and mutant kAE1, a His_6-tagged kAE1 was created. kAE1-His was transfected alone or with kAE1 901 stop. The kAE1 901 stop mutant was detectable in the eluate from the Ni^{2+} beads (Figure 7C, lane

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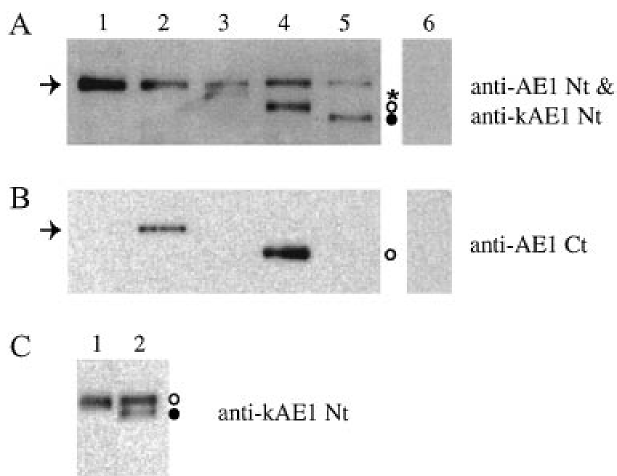

Figure 7 Co-purification of wild-type and mutant AE1 and kAE1 with AE1-His

(**A**) and (**B**) Immunoblots showing the AE1 proteins that were co-eluted with AE1-His from the Ni^{2+}-affinity resin. Extracts applied to the affinity resin were prepared from cells transfected (0.8 μg cDNA each/well) with AE1-His alone (lanes 1), AE1-His and AE1 (lanes 2), AE1-His and AE1 901 stop (lanes 3), AE1-His and kAE1 (lanes 4), AE1-His and kAE1 901 stop (lanes 5) or AE1 alone (lanes 6). The top immunoblot (**A**) was probed both anti-AE1-Nt and anti-kAE1-Nt antibodies to detect all AE1 proteins. The middle immunoblot (**B**) was probed with the anti-AE1-Ct antibody to detect AE1 and kAE1, but not AE1-His6 or the 901 stop mutants. The identity of the bands are indicated by: arrow, AE1 and AE1-His; *, AE1 901 stop; ○, kAE1; ●, kAE1 901 stop. (**C**) Immunoblot using the anti-kAE1-Nt antibody shows kAE1 proteins bound and co-eluted from the Ni^{2+}-affinity column using lysate from cells transfected with kAE1-His alone (lane 1) or kAE1-His and kAE1 901 stop (lane 2). ○, kAE1; ●, kAE1 901 stop.

2), indicating that the mutant co-purified with kAE1-His from lysate of co-transfected cells.

These co-purification results show that the 901 stop mutants interact with AE1-His and kAE1-His in transfected cells, probably as heterodimers. Hetero-oligomer formation may be the cause of the dominant negative effect resulting in intracellular retention of the wild-type protein along with the mutant protein.

DISCUSSION

A 13 bp duplication in the AE1 gene, leading to an 11-amino-acid C-terminal truncation (901 stop) of the AE1 protein, was reported in two brothers with autosomal dominant dRTA [18,25]. We report here that the 901 stop mutation causes a trafficking defect in both erythroid AE1 and kAE1 when expressed in HEK-293 cells. Co-expression of the 901 stop mutants with wild-type AE1 or kAE1 led to impaired trafficking of the wild-type proteins. This dominant negative effect mimics the situation in the cells of heterozygous individuals with autosomal dominant dRTA. The 901 stop mutants can form hetero-oligomers with AE1-His or kAE1-His, suggesting the mechanism of the dominant negative effect may be due to heterodimerization of the wild-type and mutant protein. Intracellular retention of the wild-type AE1 by interaction with the mutant AE1 would cause a marked reduction in the level of cell-surface expression of wild-type AE1. Impaired trafficking of kAE1 to the basolateral membrane in acid-secreting intercalated cells of the kidney could account for the impaired urine acidification seen in dRTA.

A recent study of dRTA patients heterozygous for the 13 bp duplication revealed a normal red cell morphology [25]. There was, however, a 35–40% reduction in the content of mutant AE1 (band 3 Walton) relative to the wild-type protein. This is consistent with our finding of impaired trafficking of erythroid AE1 as determined in transfected HEK-293 cells. The kAE1 truncation mutant was, however, retained intracellularly in transfected MDCK cells in agreement with results presented in this paper with HEK-293 cells. The severe trafficking defect seen with the kAE1 901 stop in transfected kidney-derived cells suggests that such a defect may also exist in the kidney cells of dRTA patients. The erythroid AE1 901 stop mutant had a similar trafficking defect in transfected HEK-293 cells, but was only slightly decreased in content in the red cells and exhibited normal transport in *Xenopus* oocytes [25]. This suggests that the isoform expressed (AE1 versus kAE1) and the cellular context play an important role in the phenotypic expression of the dRTA mutant. The truncation mutation in erythroid precursor cells or oocytes has a mild effect on AE1 trafficking, in contrast with the more severe defect seen in kidney cells.

A missense mutation associated with autosomal dominant dRTA, R589H, was previously shown to create a trafficking defect in kAE1, but not in erythroid AE1 [24]. Another missense mutation associated with autosomal recessive dRTA, G701D, led to impaired cell-surface expression of AE1 and kAE1 in *Xenopus* oocytes, unless the red cell protein glycophorin A was co-expressed [21]. The expression patterns of the G701D and R589H dRTA mutants provided an explanation as to why kidney function was affected, while red cell function was relatively unaffected. The kAE1 R589H mutant caused a dominant negative effect and was shown to form hetero-oligomers with wild-type in HEK-293 cells [24]. Tetramerization of wild-type and mutant aquaporin 2 was found to be the cause of autosomal dominant diabetes insipidus [38]. The ability of mutant subunits to assemble with normal subunits and impair their trafficking may be a common feature of dominant mutations in oligomeric transport proteins.

A number of published reports have shown that the C-terminal region of membrane proteins can provide important targeting information by providing specific binding sites for other proteins. Removal of the last 30 amino acids of human AE1 or the last 39 amino acids of mouse AE1 both resulted in a lack of cell-surface AE1 expression in cDNA-injected *Xenopus* oocytes [39,40]. Residues within the C-terminus of the glucose transporter 4 (GLUT4) specify the sub-endosomal destination of the protein [41–43]. Truncation of the C-terminus of other membrane proteins, such as rat brain Na^{+}/Ca^{2+} exchanger-1 (RBE-1) [44] and the glycine transporter GLYT1 [45], led to improper trafficking of the proteins. The C-terminal tail of rhodopsin contains a signal that sorts the protein to the apical membrane in polarized MDCK cells [46]. The C-terminal tail of the cystic fibrosis transmembrane conductance regulator (CFTR) interacts with the apical PDZ protein EBP50, which may stabilize or regulate CFTR [47].

AE1 contains a short C-terminal tail that ends with a class II PDZ-binding motif (X-ϕ-X-ϕ, where ϕ represents hydrophobic residues) [48]. A peptide of the C-terminal 18 amino acids of AE1 bound to a class II PDZ-domain protein, Pick-1 [49]. The 901 stop mutation eliminates this sequence from AE1 and kAE1. Efficient trafficking of AE1 and kAE1 to the plasma membrane may require interactions of the C-terminus with PDZ-domain proteins or other proteins. An acidic motif (D^{887}ADD) on the C-terminus of AE1 has also recently been shown to bind a basic region on carbonic anhydrase II [50–52], although this carbonic anhydrase II binding motif is retained in the truncation mutants. The complement of proteins interacting with AE1 in erythroid precursor cells and kAE1 in kidney cells may differ. It will be important to identify these interacting proteins and to determine how mutations in AE1 and kAE1 may affect their interactions

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with these proteins and the effect on trafficking of AE1 and kAE1.

This work was supported by a Group Grant from the Canadian Institutes of Health Research and a grant from the Kidney Foundation of Canada. We thank Jing Li for technical assistance. J. A. Q. is supported by a University of Toronto Open Scholarship.

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Received 10 April 2002/6 August 2002; accepted 13 September 2002
Published as BJ Immediate Publication 13 September 2002, DOI 10.1042/BJ20020574

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