Palmitoylation is not required for trafficking of human anion exchanger 1 to the cell surface

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AE1 (anion exchanger 1) is a glycoprotein found in the plasma membrane of erythrocytes, where it mediates the electroneutral exchange of chloride and bicarbonate, a process important in CO_2 removal from tissues. It had been previously shown that human AE1 purified from erythrocytes is covalently modified at Cys-843 in the membrane domain with palmitic acid. In this study, the role of Cys-843 in human AE1 trafficking was investigated by expressing various AE1 and Cys-843Ala (C843A) mutant constructs in transiently transfected HEK-293 cells. The AE1 C843A mutant was expressed to a similar level to AE1. The rate of N-glycan conversion from high-mannose into complex form in a glycosylation mutant (N555) of AE1 C843A, and thus the rate of trafficking from the endoplasmic reticulum to the Golgi, were comparable with that of AE1 (N555). Like AE1, AE1 C843A could be biotinylated at the cell surface, indicating that a cysteine residue at position 843 is not required for cell-surface expression of the protein. The turnover rate of AE1 C843A was not significantly different from AE1. While other proteins could be palmitoylated, labelling of transiently transfected HEK-293 cells or COS7 cells with [^{3}H]palmitic acid failed to produce any detectable AE1 palmitoylation. These results suggest that AE1 is not palmitoylated in HEK-293 or COS7 cells and can traffic to the plasma membrane.

Key words: anion exchanger 1 (AE1), biosynthesis, cysteine, membrane protein, palmitoylation.

INTRODUCTION

Palmitoylation is a common modification found in a wide variety of proteins. The modification involves a covalent linkage between palmitic acid, a 16-carbon saturated fatty acid, and the thiol group of a cysteine residue in the protein. These thioester linkages are susceptible to hydroxylamine cleavage at neutral or alkaline pH. Often palmitoylation is found in conjunction with myristoylation, which involves an amide linkage between the N-terminal glycine of a protein and the 14-carbon myristic acid. This combination of modifications is important in protein targeting (reviewed in [1]).

While fatty acylation functions as an anchor that mediates membrane binding for many extrinsic membrane proteins, intrinsic membrane proteins are anchored in the lipid bilayer by their α-helical transmembrane domains. Palmitoylation can occur in cytosolic domains, such as the C-terminal tails of G-protein-coupled receptors, or within or adjacent to the membrane-embedded domain on the cytosolic side of the membrane, with the fatty acid inserted into the lipid bilayer, as in the case of the single-spanning transferrin receptor [2]. Palmitoylation in membrane proteins is often involved in biosynthesis and function. The chemokine receptor CCR5 expressed on macrophages and lymphocytes requires palmitoylation for cell-surface targeting and stability [3,4]. Palmitoylation of caveolin-1 is required for binding and transport of cholesterol to caveolae [5] and for coupling of the c-Src tyrosine kinase [6]. Although the function of palmitoylation in many proteins has been determined, in other proteins it remains elusive [7,8].

Protein palmitoylation is mediated by PATs (palmitoyl acyltransferases), but the enzymology is poorly understood. The modification occurs post-translationally, and palmitoylating activity has been detected in the ER (endoplasmic reticulum), Golgi and plasma membrane [9–11]. A 70 kDa membrane-associated PAT was identified from human erythrocytes [9], as well as a number of other PATs from other tissues [12–16]. There is no clear consensus sequence for protein palmitoylation.

AE1 (anion exchanger 1), or band 3, is an abundant integral membrane protein found in erythrocytes. The human protein is composed of 911 amino acids and consists of two domains. The N-terminal cytosolic domain contains binding sites for the red cell cytoskeleton. The membrane domain contains 12–14 TM (transmembrane) segments and is N-glycosylated in the fourth extracellular loop at Asn-642. It carries out the bicarbonate/chloride-exchange function. CAII (carbonic anhydrase II) in the cytosol rapidly converts CO_2 into bicarbonate ions, which is removed from the red cell by AE1 into the plasma in exchange for chloride ions. AE1 and CAII form a 'metabolon' – CAII binds to the cytosolic C-terminal tail of AE1 [17–19] – and binding of CAII is necessary for efficient anion exchange [20]. The metabolon is proposed to promote substrate channeling between CAII and AE1 [21]. The kidney AE1 isoform lacks the N-terminal 65 amino acids, due to an alternative transcription start site, and is expressed in the α-intercalated cells of the distal nephron.

A number of proteins in red cells contain covalently bound fatty acids [22–24]. These fatty acids were not removed by extensive extraction with organic solvents, but were released upon treatment with neutral or alkaline hydroxylamine and subsequently identified by MS [23]. Among these acylated proteins is AE1. Interestingly, while [^{3}H]palmitic acid labelling of human red cells resulted in several proteins incorporating the label, there was little or no labelling of AE1 [25–29]. This suggests that AE1 may be stably palmitoylated and that there is little turnover of the

Abbreviations used: AE, anion exchanger; $C_{12}E_8$, octa(ethylene glycol) dodecyl ether; CAII, carbonic anhydrase II; DMEM, Dulbecco's modified Eagle's medium; ER, endoplasmic reticulum; NHS-SS-biotin, sulpho-succinimidyl 2-(biotin-amido)ethyl-1,3-dithiopropionate; PAT, palmitoyl acyltransferase; TM, transmembrane.

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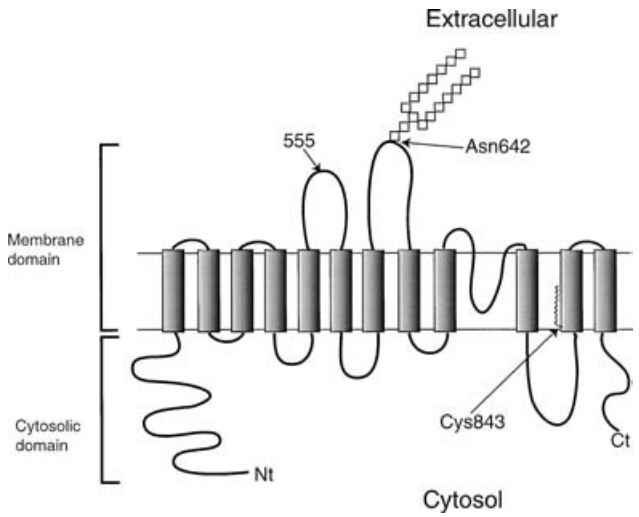

Figure 1 Folding model of human AE1

AE1 contains an N-terminal cytosolic domain and a membrane domain. The membrane domain of human AE1 consists of 12 putative transmembrane segments [33]. The endogenous N-glycosylation acceptor site (Asn-642) and palmitoylation site (Cys-843) are indicated. The position of the novel N-glycosylation acceptor site (555) in the N555 constructs is also shown. Nt, N-terminus; Ct, C-terminus.

palmitoyl group on AE1 in the red cell. This is in contrast with many cases where reversible palmitoylation may be important in stability or function of the protein [30,31].

Okubo et al. [32] identified the site of palmitoylation in human AE1 to be 69 residues from the C-terminal end in a C-terminal 8500 Da AE1 peptide purified from red cells, corresponding to Cys-843. According to current folding models of the human AE1 membrane domain [33,34], the palmitoylation site occurs near the cytosolic end of the penultimate TM segment (Figure 1). This distinguishes it from other multispanning proteins that are often palmitoylated in cytosolic loops. Cys-843 is conserved among the human anion exchanger family (AE1–3), mouse and rat AE1, and sodium–bicarbonate co-transporters, but it is not conserved in chicken AE1.

The anion-transport function of mouse AE1 in the absence of palmitoylation has been investigated [35]. The homologous Cys-861 in mouse AE1 was replaced with either methionine or serine, and the wild-type or mutant proteins were expressed in *Xenopus* oocytes. There was no significant impairment of the transport function due to the mutations. However, wild-type mouse AE1 did not incorporate [^{3}H]palmitic acid upon metabolic labelling of the oocytes. Casey et al. [36] expressed a cysteine-less human AE1 mutant in HEK-293 cells, studied its transport function in isolated microsomes, and found that anion transport was not impaired. However, HEK-293 cells transfected with the cysteine-less AE1 mutant showed a decrease in whole-cell transport rate, which was attributed to a decreased cell-surface expression of the mutant [37].

The protein content of red-cell plasma-membrane 'raft' domains has been examined [38]. The two major integral membrane protein found in the raft fraction are flotillin and stomatin (band 7), which is palmitoylated, but AE1 is absent from red cell rafts. This indicates that palmitoylation of AE1 does not serve to partition the protein into lipid rafts.

While palmitoylation does not seem to be required for anion transport, it is possible that it may be important in facilitating the folding or targeting of human AE1 during biosynthesis. This work describes several experiments using transfected cells that have been done in an attempt to investigate the role of Cys-843 palmitoylation in AE1 trafficking.

MATERIALS AND METHODS

Materials

The following is a list of materials used and their suppliers: pcDNA3 (Invitrogen, San Diego, CA, U.S.A.); QuikChange™ site-directed mutagenesis kit (Stratagene, La Jolla, CA, U.S.A.); Protein G–Sepharose (Amersham Biosciences, Baie d'Urfé, QC, Canada); DMEM (Dulbecco's modified Eagle's medium), calf serum, penicillin and streptomycin (Gibco BRL, Burlington, ON, Canada); EasyTag™ EXPRE^{35}S^{35}S protein labelling mix, [9,10-^{3}H(N)]palmitic acid and EN^{3}HANCE® autoradiography enhancer (Perkin Elmer Life Sciences, Mississauga, ON, Canada); endoglycosidase H, N-glycanase F and goat peroxidase-conjugated anti-rabbit IgG (New England Biolabs, Mississauga, ON, Canada); $C_{12}E_8$ [octa(ethylene glycol) dodecyl ether; Nikko Chemical Co., Tokyo, Japan]; chemiluminescence kit (Boehringer Mannheim, Laval, QC, Canada); ImmunoPure® immobilized streptavidin and EZ-Link™ NHS-SS-biotin [sulpho-succinimidyl 2-(biotin-amido)ethyl-1,3-dithiopropionate; MJS BioLynx, Brockville, ON, Canada]; Cy3-conjugated anti-rabbit antibody (Jackson Immunoresearch Laboratories, West Grove, PA, U.S.A.); dropping bottle with 20–30 μm filter unit (Wheaton, Millville, NJ, U.S.A.); mutagenic primers (ACGT Corp., Toronto, ON, Canada).

Site-directed mutagenesis

The entire coding sequence for wild-type human AE1 was inserted into the *Xho*I and *Bam*HI sites of pcDNA3 [33]. The AE1 N555 N-glycosylation mutant (with a novel glycosylation site at Tyr-555Asn/Val-557Thr and the mutation Asn-642Asp to eliminate the endogenous glycosylation site) was described previously [39] and was used as a template for an additional Cys-843Ala mutation. The Cys-843Ala mutation was created in wild-type AE1 and AE1 N555 constructs by the QuikChange™ system with complementary mutagenic primers to produce the AE1 Cys-843Ala (C843A) mutant and the AE1 N555 C843A double mutant. Sequencing of the constructs was carried out by the ACGT Corp.

Transient transfection and expression of AE1 mutants

HEK-293 cells were transfected by the DEAE-dextran method [40] with 1 μg of plasmid DNA per well of a 6-well plate. Cells were grown in DMEM supplemented with 10 % (v/v) calf serum, 0.5 % penicillin and 0.5 % streptomycin under air/CO_2 (19:1) at 37 °C, as described previously [33].

Enzymic deglycosylation

Cell extracts were prepared by solubilization in 1 % (v/v) $C_{12}E_8$ 48 h after transfection [33]. Insoluble material was removed by centrifugation at 14000 ***g*** for 15 min. Cell extract (50 μl) was incubated at room temperature for 1 h with no treatment, 1000 units of endoglycosidase H or 500 units of N-glycanase F; 1 vol. of 2 × Laemmli sample buffer with 4 % (w/v) SDS was added after deglycosylation.

SDS/PAGE and immunoblotting

Proteins were resolved by SDS/PAGE [41] and transferred to nitrocellulose membrane [42]. AE1 was detected with a rabbit

polyclonal antibody directed against a synthetic peptide corresponding to the C-terminal 16 residues of human AE1 [33].

Pulse–chase experiments

HEK-293 cells were transfected with 0.6 μg of plasmid DNA and grown at 37 °C for 24 h. Cells were labelled with 100 μCi/ml [^{35}S]methionine and [^{35}S]cysteine in methionine- and cysteine-free DMEM for 20 or 90 min. The radioactive medium was removed and the cells were washed once with PBS. The cells were then chased for up to 24 h (as indicated) with DMEM with 10 % calf serum and excess non-radioactive methionine. Cells were harvested at intervals by washing with PBS and solubilization in 1 × RIPA buffer (1 % deoxycholate, 1 % Triton X-100, 0.1 % SDS, 0.15 M NaCl, 10 mM Tris/HCl, pH 7.5, and 1 mM EDTA). DNA was removed by filtering using a dropping bottle with a 20–30 μm filter unit. AE1 was immunoprecipitated with 3 μl of anti-AE1 C-terminal antibody followed by 30 μl of Protein G–Sepharose. Proteins were eluted with 50 μl of 2 × SDS sample buffer at room temperature for 10 min. Proteins were resolved by SDS/PAGE and detected by autoradiography.

Cell-surface biotinylation

The procedure for biotinylation has been described previously [39]. Cells were treated twice with 1 ml of 0.8 mM EZ-Link™ NHS-SS-biotin for 15 min at room temperature. Biotinylated proteins in cell extracts were captured using streptavidin beads. The presence of AE1 in the total, unbound and bound fractions was detected by immunoblotting.

[^{3}H]Palmitic acid incorporation

HEK-293 or COS7 cells grown in 35 mm plates were transfected with 0.6 μg of plasmid DNA per plate as described above. At 24 h after transfection, cells were washed with PBS and incubated in serum-free DMEM with penicillin and streptomycin for an additional 24 h. Cells were then washed with PBS and labelled with [^{3}H]palmitic acid at 37 °C for 2 h or 6 h [43]. [^{3}H]Palmitic acid, supplied in ethanol, was dried under a stream of nitrogen gas and redissolved in DMSO. The final composition of the labelling medium was 1 % DMSO (containing 0.35 mCi of [^{3}H]palmitic acid) and antibiotic-free DMEM supplemented with 1 % calf serum. The final radioactivity of the medium was 0.5 mCi/ml. To verify expression of the protein, a separate batch of cells was incubated with methionine- and cysteine-free DMEM containing 10 μCi/ml [^{35}S]methionine/cysteine for 2 h. Proteins were immunoprecipitated as described above. Proteins were resolved with SDS/PAGE. The SDS gels were subsequently treated with 1 M Tris/HCl (pH 7.5) or 1 M hydroxylamine (pH 7.5) overnight, fixed, then treated with EN^{3}HANCE® autoradiography enhancer. Autoradiographs were typically exposed for 1 month to reveal [^{3}H]palmitic acid labelling.

RESULTS

C843A mutants expressed well in transfected cells

In order to study the importance of palmitoylation in AE1 biosynthesis, site-directed mutagenesis was carried out to remove the palmitoylation site (C843A) in various human AE1 constructs. These constructs were then expressed in HEK-293 cells, and AE1 expression was analysed by immunoblotting. The expression level of AE1 C843A is not significantly different from that of wild-type AE1 (98 ± 26 % of wild-type, $n = 6$; Figure 2). The C843A mutant therefore is not unstable or targeted for rapid degradation.

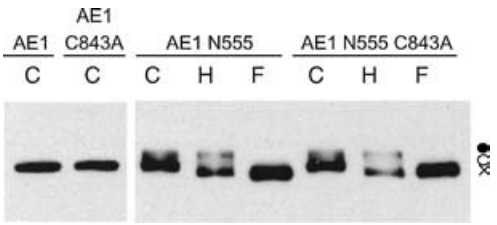

Figure 2 Expression of AE1 constructs in HEK-293 cells

Immunoblots of cell extracts prepared from HEK-293 cells transiently transfected with AE1, AE1 C843A, AE1 N555 or AE1 N555 C843A. Expression of proteins was verified by immunoblotting using an antibody directed against the C-terminus of AE1. Cell lysates had been treated with endoglycosidase H (H), peptide N-glycanase F (F) or not treated (C) at room temperature for 1 h. ●, The position of AE1 bearing complex N-glycan; ○, the position of high-mannose glycoprotein; ×, the position of the deglycosylated protein.

Membrane proteins are usually co-translationally N-glycosylated and the N-glycan is processed during trafficking. High-mannose N-glycans are found in the ER to the *cis*-Golgi, and are converted into a complex form as the protein moves through the medial Golgi. AE1 contains a single site of N-glycosylation at Asn-642. The oligosaccharide at this position is not processed to a complex form in transfected HEK-293 cells, although the protein traffics to the cell surface [39]. Cell-surface biotinylation studies [20,39] showed that approx. 30 % of AE1 was at the cell surface in transiently transfected HEK cells. Moving the N-glycosylation acceptor site to the preceding extracellular loop at Asn-555 (AE1 N555) permits efficient processing to occur. The complex N-glycosylated fraction was expressed at the transfected HEK-293 cell surface as shown by biotinylation [39] (see also Figure 4, below) and represented approx. 30 % of the total AE1 protein [39]. Thus the amount of AE1 and AE1 N555 found in the plasma membrane is very similar [39]. A double N-glycosylation mutant that contained the N555 mutation and the endogenous site at Asn-642 acquired a complex oligosaccharide at Asn-555 and a high-mannose oligosaccharide at Asn-642 [39]. This shows that even though the protein had trafficked to the Golgi (as evidenced by processing of the N555 site), the endogenous site retained its high-mannose structure. In addition, a Tyr-555Cys mutant in a cysteine-less background expressed in HEK-293 cells could be biotinylated at the cell surface and was functional in anion transport [34,37,44], indicating that mutation of the tyrosine residue at position 555 does not significantly alter trafficking or folding of the protein.

The use of the N555 mutants allows a convenient assessment of the trafficking status of various mutants in cells [45–47]. Expression of AE1 N555 in HEK-293 cells resulted in two bands on the immunoblot (Figure 2), in agreement with previous studies [39]. The lower band was sensitive to endoglycosidase H treatment and corresponds to high-mannose glycosylated AE1. The upper band (approx. 30 % of total protein) was resistant to endoglycosidase H and contains complex N-glycan. When the mutant AE1 N555 C843A was expressed in HEK-293 cells, the protein also acquired complex N-glycan (about 30 % of total protein), suggesting that the Cys-843Ala mutation did not impair trafficking. The presence of the complex N-glycan shows that AE1 N555 C843A had moved from the ER to the medial Golgi, similar to AE1 N555.

In order to investigate whether the Cys-843Ala mutation could impair the trafficking of AE1 through the ER–Golgi pathway, a [^{35}S]methionine pulse–chase experiment was carried out to

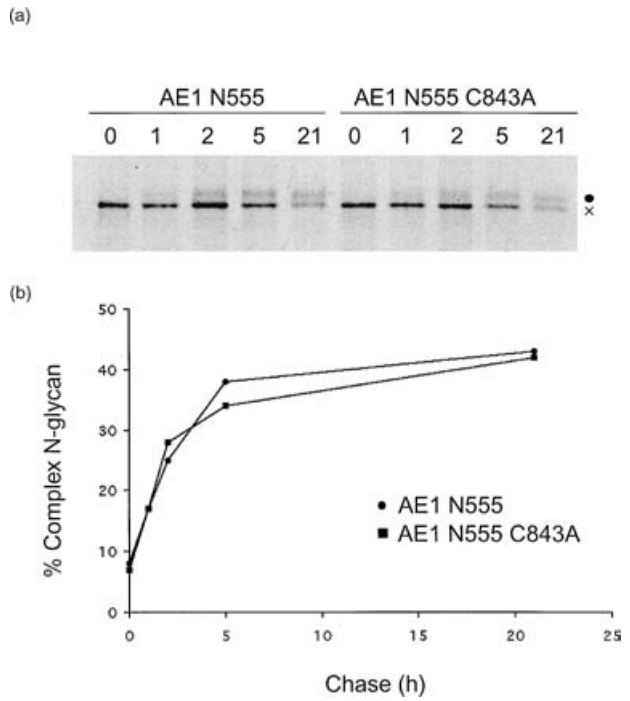

Figure 3 Pulse–chase experiment showing processing of the N555 N-glycan

(**a**) Autoradiograph showing conversion of the AE1 N555 N-glycan from high-mannose into complex form. HEK-293 cells transiently transfected with AE1 constructs were labelled with [^{35}S]methionine and [^{35}S]cysteine for 20 min at 37 °C, and then chased for 21 h. Cells were harvested at intervals and AE1 proteins immunoprecipitated with an anti-C-terminal antibody. To resolve better the high-mannose and complex glycoproteins, samples were treated with endoglycosidase H before SDS/PAGE. ●, The position of protein bearing complex N-glycan; ×, the position of the deglycosylated high-mannose protein. (**b**) Quantification of the N-glycan conversion of AE1 N555 and AE1 N555 C843A. The percentage of protein bearing complex N-glycan is calculated as the signal of the upper band divided by the sum of the signals of the upper and lower bands in the autoradiograph.

follow the conversion of the high-mannose N-glycan into complex N-glycan as the proteins trafficked from the ER to the Golgi complex. The resulting autoradiograph is shown in Figure 3(a), and the percentage of polypeptide bearing complex N-glycan relative to total signal at each time point is quantified in Figure 3(b). The kinetics of the N555 N-glycan conversion were not altered due to the Cys-843Ala mutation. This suggests that the Cys-843Ala mutation does not affect the rate of trafficking of AE1 from the ER to the medial Golgi.

AE1 C843A was expressed on the surface of transfected cells

To determine whether AE1 C843A could traffic to the cell surface, cell-surface biotinylation experiments were performed using a membrane-impermeant biotinylation reagent, NHS-SS-biotin. The cell lysates were separated into unbound (S) and bound (B) fractions after addition of streptavidin beads, and the fractions were analysed by immunoblotting against AE1 (Figure 4). In the absence of NHS-SS-biotin, no AE1 band was detected in the streptavidin-bead-bound (B) fraction. Both wild-type AE1 and AE1 C843A were present in the streptavidin-bead-bound fraction, and thus were expressed on transfected HEK-293 cell surface. The polypeptides of AE1 N555 and AE1 N555 C843A bearing complex N-glycan (upper band) had also trafficked to the cell surface and could be specifically biotinylated, while the high-mannose polypeptides (lower band) represented the intracellular

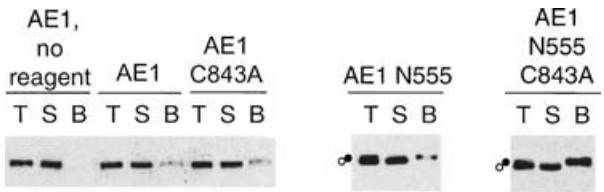

Figure 4 Cell-surface biotinylation of AE1 proteins

HEK-293 cells were transiently transfected with AE1 constructs. Post-transfection (2 days), cell-surface biotinylation was carried out with the membrane-impermeant reagent NHS-SS-biotin. Negative control experiments (AE1, no reagent) were carried out in the absence of NHS-SS-biotin. Total cell lysate (T), supernatant not bound by streptavidin beads (S) and the fraction bound by streptavidin beads (B) were subjected to immunoblotting with an antibody directed against the C-terminus of AE1. ●, The position of protein bearing complex N-glycan; ○, the position of protein bearing high-mannose N-glycan.

pools and were not biotinylated (Figure 4). The biotinylated AE1 and AE1 C843A proteins often exhibited a decreased mobility in SDS/PAGE compared with the total and unbound proteins. The change in mobility does not appear to be a result of the N-glycan being processed to a complex structure, unlike the case for the N555 samples. The biotinylated proteins were susceptible to endoglycosidase H treatment (results not shown), indicating that the N-glycans on biotinylated AE1 or AE1 C843A had a high-mannose structure, similar to the total and unbound proteins. We speculate that modification of the AE1 protein by N-hydroxysulphosuccinimide ester at various lysine residues caused a change in the hydrodynamic shape of the protein, and thus its mobility in SDS/PAGE. Immunolocalization of AE1 and AE1 C843A in transfected HEK cells (results not shown) showed that both proteins were expressed on the cell surface, in agreement with the biotinylation results.

Turnover rate of AE1 was not affected by Cys-843Ala mutation

Pulse–chase experiments were carried out to examine the biosynthesis and stability of AE1 C843A in HEK-293 cells. Figure 5(a) shows a representative autoradiograph and Figure 5(b) shows the percentage of protein remaining during the chase period. With a pulse period of 20 min, there was a consistently observed increase in signals immunoprecipitated by the anti-AE1 C-terminal antibody at 2 h chase, relative to the 0 h time point, in both AE1 and AE1 C843A constructs. The peak probably represented the completion of AE1 synthesis. A 90-min pulse greatly reduced the height of the peak (results not shown), since the longer pulse period allowed a larger proportion of AE1 protein to be completely synthesized and immunoprecipitated by the anti-C-terminal antibody at 0 h chase. Over the 27 h chase period, the amount of AE1 and AE1 C843A exhibited similar patterns of increase and decrease ($n = 3$). With the amount of signal at 0 h chase set at 100 %, the half-life of AE1 was approx. 13 h, consistent with previous observations [45]. The half-life of AE1 C843A was about 12 h, very similar to AE1. This suggests that the Cys-843Ala mutation does not significantly affect the turnover rate of AE1.

AE1 was not palmitoylated in HEK-293 or COS7 cells

In order to ascertain the palmitoylation status of wild-type AE1 and AE1 C843A expressed in transfected cells, palmitic acid incorporation studies were carried out. Transiently transfected HEK-293 (Figure 6a) or COS7 cells (Figure 6b) were labelled with [^{3}H]palmitic acid for 2 h and solubilized. AE1 or AE1

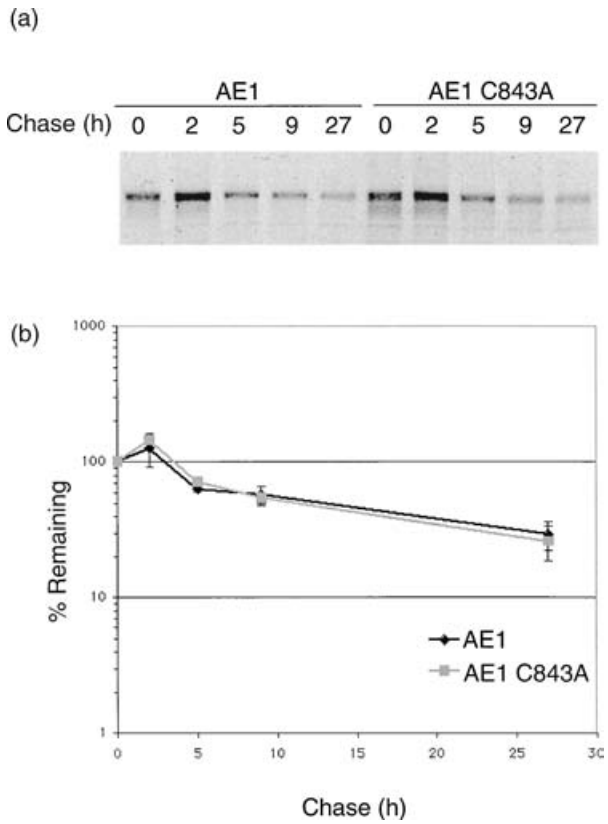

Figure 5 Stability of AE1 and AE1 C843A expressed in HEK-293 cells

(**a**) Autoradiograph showing pulse–chase results of AE1 and AE1 C843A. HEK-293 cells were transiently transfected with the constructs and labelled with [^{35}S]methionine and [^{35}S]cysteine for 20 min at 37 °C, and then chased for 27 h. Cells were harvested at intervals and AE1 proteins immunoprecipitated with an anti-C-terminal antibody. (**b**) Quantification of signals from pulse–chase experiments ($n = 3$).

C843A was immunoprecipitated and analysed by SDS/PAGE and autoradiography (Figure 6). Multiple tritium-labelled bands were present in total cell lysates of both HEK-293 (Figure 6a) and COS7 cells (Figure 6b, left-hand panel). These bands disappeared upon treatment with neutral hydroxylamine (Figure 6b, right-hand panel), indicating that the labelled palmitic acid was attached by thioester linkages and not due to incorporation of metabolized [^{3}H]palmitic acid. The presence of these bands shows that HEK-293 and COS7 cells were capable of palmitic acid uptake from the medium, as well as carrying out palmitoylation of certain proteins. However, no label was detected in the immunoprecipitate of AE1 or AE1 C843A even after prolonged exposure of the autoradiographs. Labelling transfected HEK-293 cells with [^{3}H]palmitic acid for 6 h resulted in a band in the immunoprecipitate fractions of both AE1 and AE1 C843A which was not removed by hydroxylamine treatment, suggesting that the labels were due to incorporation of [^{3}H]palmitic acid metabolites during the prolonged labelling period (results not shown). No tritium-labelled AE1 bands were observed in the immunoprecipitate fractions of transfected LLC-PK$_1$ cells (results not shown). These results show that no detectable palmitoylation of AE1 was observed in three different transfected cell lines.

DISCUSSION

The lack of detectable palmitoylation of AE1 expressed in HEK-293 or COS7 cells was surprising, as both cell lines had been used previously in studying the palmitoylation of other proteins [48] and thus were clearly capable of fatty acid uptake and incorporation into other proteins (Figure 6). Lack of palmitoylation was also observed with mouse AE1 in *Xenopus* oocytes [35]. The lack of AE1 palmitoylation in these cells could be due to a number of reasons. It is possible that AE1 is not palmitoylated at Cys-843. However, this is unlikely, as a C-terminal AE1 peptide containing a palmitic acid had been isolated from human red cells [32], and the presence of a covalently linked fatty acid in AE1 had been demonstrated in several previous experiments [22–24]. The AE1 palmitoylation site may be uniquely recognized by a palmitoyl acyltransferase present in erythrocytes or erythroid precursors but absent from HEK-293, COS7 cells and oocytes. Alternatively, there may be differences in the conformation of heterologously expressed AE1 that results in its failure to be palmitoylated. The biosynthetic environment

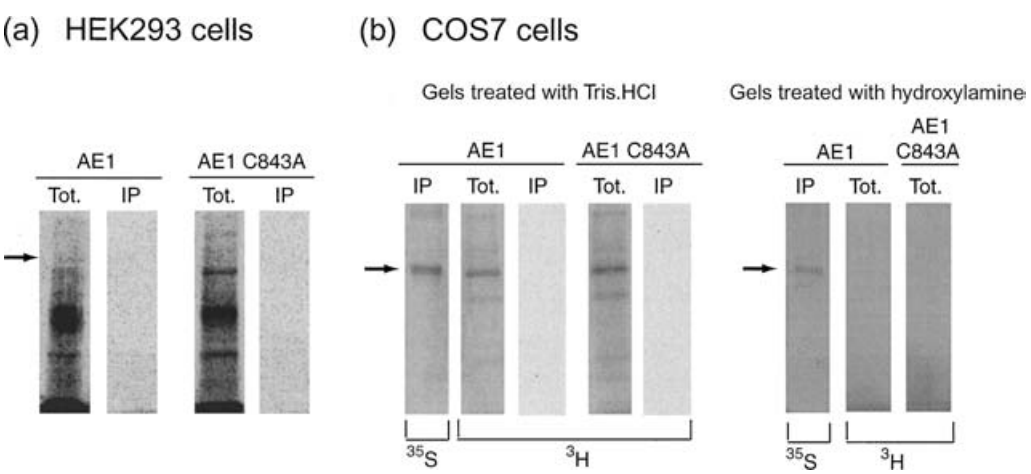

Figure 6 [^{3}H]Palmitic acid labelling of transiently transfected HEK-293 and COS7 cells

HEK-293 cells (**a**) or COS7 cells (**b**) transiently transfected with AE1 or AE1 C843A were labelled with [^{3}H]palmitic acid or [^{35}S]methionine/cysteine at 37 °C for 2 h. Cells were then harvested. An aliquot of the total cell lysates (Tot.) was obtained. AE1 proteins were immunoprecipitated (IP) using an antibody directed against the C-terminus of AE1. (**a**) Transfected HEK-293 cells labelled with 0.5 mCi/ml [^{3}H]palmitic acid. Gel was not treated with Tris/HCl or hydroxylamine. Arrow indicates approximate position of AE1 on the autoradiograph. (**b**) Transfected COS7 cells labelled with 0.5 mCi/ml [^{3}H]palmitic acid (^{3}H) or 10 μCi/ml [^{35}S]methionine/cysteine (^{35}S). Left-hand panel: autoradiograph from gel treated with 1 M Tris/HCl, pH 7.5. Right-hand panel: autoradiograph from gel treated with 1 M hydroxylamine, pH 7.5. Arrows indicate the position of AE1.

of AE1 in kidney cells and oocytes is different from that in erythroid precursor cells, as a number of erythrocyte proteins such as ankyrin and glycophorin A, which interact with AE1, are not found in kidney cells or oocytes. These proteins may be required to induce subtle changes in the conformation or changes in the oligomeric state of AE1 in order for it to achieve palmitoylation. This also raises the question of whether the kidney AE1 isoform is physiologically palmitoylated. Acylation of AE1 may occur at the level of the plasma membrane. *In vitro*, spontaneous non-enzymic acylation of cysteine residues has been reported [49–51], although it is not clear whether this can occur *in vivo*. In cells, fatty acyl-CoA is sequestered by acyl-CoA-binding proteins, thus the concentration of acyl-CoA available for spontaneous reaction is very low. The half-time of this spontaneous reaction *in vivo* has been estimated to be in the order of several tens of hours [52]. However, the average erythrocyte survives for about 120 days. It is possible that during this long period of time AE1 in the plasma membrane may be acylated spontaneously. The limited time in the set-up of our experiment, or in the oocyte experiment [35], does not allow this process to occur, even if it exists. If AE1 is indeed autopalmitoylated at the plasma membrane after biosynthesis, the modification is not expected to be necessary for the trafficking of the protein.

The addition of the palmitoyl group on TM11 may alter the conformation of AE1 in this region even after biosynthesis. Fluorescence and CD spectroscopic studies [53] indicated that the presence of palmitic acid within the membrane domain of a protein can affect conformation of the surrounding peptides. Palmitoylation of TM segments may serve to tilt it from an orientation perpendicular to the plane of the bilayer. While palmitoylation was not sufficient to force a hydrophilic segment into a hydrophobic micellar environment, when present in a TM segment it increased the helical content of the peptide [54]. The absence of the homologous cysteine residue and thus the palmitoyl group from chicken AE1 suggests that palmitoylation is not essential for AE1 structure or transport function.

It remains possible that palmitoylation is important in the biosynthesis of AE1 or other processes not examined in the present study. The issue must be addressed in the future with the use of a cell line capable of supporting AE1 palmitoylation, or by co-transfection with erythrocyte PAT, which may be a candidate enzyme for this modification. Other issues to be examined include whether palmitoylation has a role in AE1 interaction with other proteins. It appears that the AE1–CAII interaction is not severely affected by lack of AE1 palmitoylation, as HEK-293 cells were employed to study this interaction [20]. Also, the cytosolic C-terminal tail of AE1 fused to glutathione S-transferase was sufficient to bind to CAII in microtitre plate binding assays [17]. The C-terminal portion of the membrane domain possibly plays a role in transport [55,56]. The effect of palmitoylation on the anion-transport function of AE1 should be re-examined in cells capable of palmitoylating AE1. Although the lack of AE1 palmitoylation may not be detrimental to its transport function when expressed in HEK-293 or COS7 cells, topological studies of the C-terminal end of the membrane domain done in these cell lines should be interpreted with care, since the lack of palmitoylation of Cys-843 may imply greater topological flexibility than may occur if Cys-843 were palmitoylated as in the mature red blood cell.

We thank Dr Emmanuelle Cordat for assistance in confocal microscopy and Jing Li for help in the pulse–chase experiments.

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Received 6 June 2003/25 November 2003; accepted 27 November 2003
Published as BJ Immediate Publication 27 November 2003, DOI 10.1042/BJ20030847

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