Transmembrane folding of the human erythrocyte anion exchanger (AE1, Band 3) determined by scanning and insertional N-glycosylation mutagenesis

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The human erythrocyte anion exchanger (AE1, Band 3) contains up to 14 transmembrane segments, with a single site of N-glycosylation at Asn⁶⁴² in extracellular (EC) loop 4. Scanning and insertional N-glycosylation mutagenesis were used to determine the folding pattern of AE1 in the membrane. Full-length AE1, when expressed in transfected human embryonic kidney (HEK)-293 or COS-7 cells, retained a high-mannose oligosaccharide structure. Scanning N-glycosylation mutagenesis of EC loop 4 showed that N-glycosylation acceptor sites (Asn-Xaa-Ser/Thr) spaced 12 residues from the ends of adjacent transmembrane segments could be N-glycosylated. An acceptor site introduced at position 743 in intracellular (IC) loop 5 that could be N-glycosylated in a cell-free translation system was not N-glycosylated in transfected cells. Mutations designed to disrupt the folding of this loop enhanced the level of N-glycosylation at Asn⁷⁴³ *in vitro*. The results suggest that this loop might be transiently exposed to the lumen of the endoplasmic reticulum during biosynthesis but normally folds rapidly, precluding N-glycosylation. EC loop 4 insertions into positions 428, 484, 754

and 854 in EC loops 1, 2, 6 and 7 respectively were efficiently N-glycosylated, showing that these regions were extracellular. EC loop 4 insertions into positions 731 or 785 were poorly N-glycosylated, which was inconsistent with an extracellular disposition for these regions of AE1. Insertion of EC loop 4 into positions 599 and 820 in IC loops 3 and 6 respectively were not N-glycosylated in cells, which was consistent with a cytosolic disposition for these loops. Inhibitor-affinity chromatography with 4-acetamido-4'-isothiocyanostilbene-2,2'-disulphonate (SITS)-Affi-Gel was used to assess whether the AE1 mutants were in a native state. Mutants with insertions at positions 428, 484, 599, 731 and 785 showed impaired inhibitor binding, whereas insertions at positions 754, 820 and 854 retained binding. The results indicate that the folding of the C-terminal region of AE1 is more complex than originally proposed and that this region of the transporter might have a dynamic aspect.

Key words: biosynthesis, glycoprotein, membrane protein, processing, topology.

INTRODUCTION

Band 3, the anion exchanger (AE1) of human erythrocytes, is a 911-residue glycoprotein that consists of two structurally and functionally distinct domains [1–6]. The N-terminal domain (residues 1–359) is located in the cytosol, where it provides the anchoring point for the erythrocyte cytoskeleton and other cytosolic proteins [7]. The C-terminal membrane domain (residues 360–911) is responsible for the anion-exchange function [8–12]. Current models for the transmembrane (TM) organization of this membrane protein based on hydropathy analysis and experiments with vectorial labelling, proteolysis and antibody binding indicate that the polypeptide chain might span the membrane up to 14 times [5,6,13]. Human AE1 contains a single site of N-glycosylation at Asn⁶⁴² [1,2,14], which provides an endogenous marker for the extracellular disposition of extracellular (EC) loop 4.

In a previous study we examined the topology of human AE1 by mutating the endogenous N-glycosylation site (N642D) and introducing novel sites (Asn-Xaa-Ser/Thr) into hydrophilic loops connecting TM segments [15]. N-glycosylation of these sites was determined by cell-free translation of the membrane domain of

AE1 in a reticulocyte lysate in the presence of microsomal membranes. Scanning N-glycosylation mutagenesis of EC loop 4 showed a strict distance dependence for the N-glycosylation of loops in polytopic membrane proteins such as AE1. Short loops were not N-glycosylated and sites in EC loop 4 had to be located more than 12 residues from the end of the preceding TM segment and more than 14 residues away from the beginning to the following TM segment to be N-glycosylated *in vitro*. Acceptor sites located from residues 735 and 750 could be N-glycosylated, suggesting that this entire region was exposed to the lumen of the microsomes during cell-free translation. Lys⁷⁴³ can, however, be cleaved by trypsin trapped within resealed ghosts, showing that this trypsin-sensitive loop (T-loop) of AE1 faces the cytosolic side of the erythrocyte membrane [16]. It is possible that the T-loop is exposed transiently to the lumen of the endoplasmic reticulum during biosynthesis and that it normally folds into the protein to be exposed to the cytosolic side of the membrane in the final folded state. The N-glycosylation results were most consistent with a 12 TM model that included a membrane-spanning T-loop as shown in Figure 1.

The topology of membrane proteins derived from N-glycosylation mutagenesis and cell-free translation have given results that

Abbreviations used: C₁₂E₈, octaethylene glycol mono-n-dodecyl ether; DIDS, 4,4'-di-isothiocyanostilbene-2,2'-disulphonate; EC loop, extracellular loop; HEK, human embryonic kidney; IC loop, intracellular loop; SITS, 4-acetamido-4'-isothiocyanostilbene-2,2'-disulphonate; TM, transmembrane.

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Figure 1 Folding model of the membrane domain of human AE1 containing 12 TM segments and a membrane-spanning IC loop 5 (T-loop)

Extracellular loops are numbered EC 1–7 and intracellular loops are numbered IC 1–6. The single site of N-glycosylation is located at Asn⁶⁴² in EC loop 4. The positions of the EC loop 4 insertions are indicated by large triangles. Abbreviations: Nt, N-terminus; Ct, C-terminus.

do not always agree with the topology determined in intact cells [17,18]. We therefore expressed human AE1 N-glycosylation mutants in human embryonic kidney (HEK)-293 and COS-7 cells to determine the status of the N-glycosylation sites *in vivo*. The N-glycosylation patterns of AE1 mutants in cells were similar but not identical to the situation observed by cell-free translation. Importantly, an AE1 mutant containing an acceptor site at position 743 that was N-glycosylated *in vitro* did not result in the production of an N-glycosylated protein when expressed in transfected cells. The orientation of short loops in AE1 was studied by the insertion of EC loop 4 into these hydrophilic regions and expression of the insertion mutants in transfected cells. The N-glycosylation pattern led us to refine the folding model of AE1.

An important consideration in any studies designed to test the topology of membrane proteins by using mutagenesis and expression is whether the expressed protein is properly folded and functional. AE1 is inhibited by stilbene disulphonates that bind to an external site on the protein [19]. 4-Acetamido-4'-isothiocyanostilbene-2,2'-disulphonate (SITS)-Affi-Gel is an inhibitor-affinity resin that was developed to purify anion exchangers [20,21]. The binding of AE1 mutants to the immobilized inhibitor provides a convenient assay for the native state of the expressed proteins. We used this resin to determine the effect of insertions on the structure of AE1 and found that insertions into

certain loops prevented inhibitor binding. This suggests that integrity of these loops is required for the proper protein folding.

EXPERIMENTAL

Materials

Materials were purchased from the following companies (in parentheses): pcDNA3 (Invitrogen); Transformer[™] site-directed double-stranded mutagenesis system (Clontech); concanavalin A, protease inhibitors (Sigma Chemical Co.); concanavalin A-Sepharose, T7 sequencing kit and restriction enzymes (Pharmacia Biotech Inc.); cell culture media (Life Technologies); Modulis[™] *in vitro* transcription/translation system and dog pancreatic microsomes (MBI Fermentas); endoglycosidase H₁, N-glycanase F and goat peroxidase-conjugated anti-rabbit IgG (New England Biolabs); octaethylene glycol mono-n-dodecyl ether (C₁₂E₈) (Nikkol Chemical Co.); chemiluminescence kit (Boehringer Mannheim); SQ-EASY[™] diagnostic filters, 25 μ m particle retention (Porex). SITS-Affi-Gel was prepared as described previously [20]. A polyclonal antibody directed against a synthetic peptide corresponding to the C-terminal 16 residues of human AE1 was prepared by injection of the peptide conjugated to keyhole limpet haemocyanin into rabbits (SynPep, Dublin, CA, U.S.A.).

Site-directed mutagenesis

The entire coding sequence for human AE1 was inserted into the *Xho*I and *Bam*HI sites of pcDNA3, which had been modified to eliminate superfluous restriction sites from the multiple cloning site. This vector permits the expression of AE1 mutants in either a cell-free system or transfected cells. Oligonucleotide-directed mutagenesis was performed with the double-stranded mutagenesis system from Clontech. All mutations and insertions were confirmed by DNA sequencing [22]. The endogenous N-glycosylation site, Asn⁶⁴², in AE1 was mutated to Asp (N642D) to produce the non-glycosylated protein [15]. Insertion mutants were constructed by ligation of a PCR product encoding residues 626–659 of EC loop 4 into novel *Xba*I sites at positions 428, 484, 599, 731, 754, 785, 820 and 854, as described previously [15]. With the insertion at position 731 as an example, the resulting amino acid sequences at the insertion sites had the form ... Arg⁷³⁰ - Leu⁷³¹ - **Asp**⁶²⁶ - **Thr**⁶²⁷ ... **Glu**⁶⁵⁸ - **Phe**⁶⁵⁹ - Leu⁷³¹ - **Asp**⁷³² - Thr⁷³³ ... The residues in bold represent the inserted EC loop 4 sequence and the underlined residues are those that were altered or duplicated by the creation of the *Xba*I site. SAO AE1, with a nine-residue deletion (Ala⁴⁰⁰-Ala⁴⁰⁸), was made by using a PCR deletion strategy [23] and the following flanking oligonucleotides: forward deletion primer, 5'-GACATCACAGATGTCATCTT-CATCTACTTTGC-3'; reverse deletion primer, 5'-GAAGATG-ACATCTGTGATGTCCTCAGG-3'; forward primer, 5'-CC-TGCCAAGCCAGACTCC-3'; reverse primer, 5'-CCACCAG-CAGGATGAGCC-3'. Mutations in AE1 are listed in Table 1.

Cell-free translation of AE1 mutants

Transcription and translation of AE1 mutants *in vitro* were performed by using rabbit reticulocyte lysates supplemented with dog pancreatic microsomes, as described previously [15].

Expression of AE1 mutants in transfected cells

HEK-293 and green-monkey kidney COS-7 cells were grown in Dulbecco's modified Eagle's medium supplemented with 4 mM glutamine, 10% (v/v) fetal calf serum, 0.5% penicillin and 0.5% streptomycin under air/CO₂ (19:1) at 37 °C. Cells were transfected with 5 µg of plasmid DNA per 100 mm diameter plate by the DEAE-dextran method [24] and grown for 48 h. Cells were washed several times with ice-cold PBS. Cell extracts (5–10 mg/ml protein) were prepared by solubilizing each plate of washed cells at 0 °C in 1 ml of solubilizing buffer [1% (v/v) Triton X-100 or C₁₂E₈/150 mM NaCl/5 mM sodium phosphate (pH 7.4)] supplemented with 1 mM PMSF, 0.5 µg/ml leupeptin and 0.7 µg/ml pepstatin, followed by centrifugation at 14000 g (12000 rev./min) for 15 min to remove insoluble material. Extracts were also prepared by solubilizing the washed cells directly with Laemmli sample buffer and filtering the extract through SQ-EASY pre-filters to remove DNA.

At 2 days after transfection, COS-7 cells grown in a 100 mm diameter dish were labelled with 100 µCi/ml of [³⁵S]methionine for 90 min. Labelled cells were washed with PBS and lysed in 1.5 ml RIPA buffer [1% deoxycholate/1% (v/v) Triton X-100/0.1% SDS/150 mM NaCl/1 mM EDTA/10 mM Tris/HCl (pH 7.5)] supplemented with protease inhibitors at 0 °C. AE1 was immunoprecipitated overnight with 5 µl of a rabbit polyclonal antibody directed against the C-terminal 16 residues of human AE1, followed by incubation for 2–4 h with 20 µl of Protein G-Sepharose. Beads were washed three times with RIPA buffer and bound proteins were solubilized with 50 µl of 2 × Laemmli sample buffer [4% (w/v) SDS] at room temperature.

Table 1 N-glycosylation mutants in human AE1

Abbreviations: 428a, no N-glycosylation acceptor sites in either endogenous or inserted EC4 loops; 428b, an N-glycosylation acceptor site (N642SS) in endogenous EC4 loop but none in inserted EC4 loop; 428c, an N-glycosylation acceptor site (N642'SS) in inserted EC4 loop but none in endogenous EC4 loop; 428d, an N-glycosylation acceptor site in both endogenous and inserted EC4 loop; +, N-glycosylated; –, not N-glycosylated; (+), low level (less than 20%) N-glycosylated.

Variant	N-glycosylation site	N-glycosylation (%)	
		<i>In vivo</i>	<i>In vitro</i>
Endogenous site	N642SS	63 ± 4 (4)	46 ± 6 (3)
EC4 mutations			
T627N	N627YT	0 (3)	0
K631N, L632S	N631SS	0 (3)	0
L632N, V634S	N632SS	0 (3)	0
S633N, P635S	N633VS	40 ± 3 (3)	44
V634N, P635S, D636S	N634SS	0 (3)	0
P635N, G637S	N635DS	50 ± 7 (3)	0
P635L, D636N, F638S	N636GS	48 ± 5 (3)	11
G637N, K639S	N637FS	59 ± 2 (3)	45
F638N, V640S	N638KS	62 ± 3 (3)	40
K639N	N639VS	59 ± 3 (3)	51
S643N, A645S	N643SS	61 ± 3 (3)	53
S644N, R646S	N644AS	63 ± 3 (3)	64
A645N, G647S	N645RS	63 ± 3 (3)	46
R646N, W648S	N646GS	67 ± 5 (3)	53
G647N, W648S, V649S	N647SS	66 ± 3 (3)	51
W648N, I650S	N648VS	58 ± 5 (3)	51
V649N, H651S	N649IS	0 (3)	0
I650N, P652S	N650HS	0 (3)	0
L655N	N655RS	0 (3)	0
IC5 mutation			
K743N	N743AS	0 (3)	29
EC4 insertion mutations			
Position 428 in EC1 (428a)	None	—	—
Position 428 in EC1 (428b)	N642SS	+	+
Position 428 in EC1 (428c)	N642'SS	+	+
Position 428 in EC1 (428d)	N642'SS, N642SS	+, +	+, +
Position 484 in EC2	N642'SS	+	+
Position 599 in IC3	N642'SS	—	—
Position 731 in IC5	N642'SS	(+)	+
Position 754 in IC5	N642'SS	+	+
Position 785 in EC6	N642'SS	(+)	+
Position 820 in IC6	N642'SS	—	(+)
Position 854 in EC7	N642'SS	+	+

Samples were resolved by SDS/PAGE and radiolabelled proteins were detected by autoradiography.

SDS/PAGE and immunoblotting

AE1 was detected by immunoblotting of cell extracts. In brief, proteins were resolved by SDS/PAGE [25] and transferred to nitrocellulose [26]. AE1 was detected with a rabbit polyclonal antibody (1/10000 dilution of serum) directed against a synthetic peptide corresponding to the C-terminal 16 residues of human AE1 [1,2]. Blots were incubated with HRP-conjugated goat anti-rabbit secondary antibody (1/5000 dilution). The blot was developed by chemiluminescence and exposure to X-ray film.

Lectin gel-shift assay

A lectin gel-shift assay was developed to increase the separation of N-glycosylated and non-glycosylated forms of AE1 on SDS/polyacrylamide gels. The direct addition of concanavalin A to

10 mg/ml to the samples before the addition of Laemmli sample buffer provided a better separation of the bands. Samples were not boiled and were applied to the SDS/polyacrylamide gel without delay. A superior separation was achieved by including concanavalin A at 10 mg/ml in the top portion (0.25–0.5 cm) of the separating gel. This gel was prepared by applying a second separating gel solution containing concanavalin A above a shortened polymerized separating gel. After polymerization of the gel in the presence of concanavalin A, a normal stacking gel was prepared. It was essential to include 0.25 M methyl α -D-mannopyranoside in the immunoblotting solutions to prevent non-specific binding of immunoglobulins to the concanavalin A. This addition was not required for [35 S]methionine-labelled proteins prepared by cell-free translation or immunoprecipitated from radiolabelled transfected cells.

Enzymic deglycosylation

Cell extracts (50 μ l) prepared in 1% Triton X-100 were treated with 1000 units/ml endoglycosidase H_t or 500 units/ml N-glycanase F at 37 °C for 1 h followed by the addition of 1 vol. of 2 $\times$ Laemmli sample buffer containing 4% (w/v) SDS.

Concanavalin A–Sephacrose binding

Aliquots (50 μ l) of the cell extract in 1% (v/v) Triton X-100 were incubated at 4 °C for 1 h with 10 μ l of concanavalin A–Sephacrose equilibrated in 0.1% (v/v) Triton X-100/500 mM NaCl/20 mM Tris/HCl (pH 7.4) containing final concentrations of 1 mM MgCl₂, 1 mM CaCl₂ and 1 mM MnCl₂ in the presence or absence of 0.25 M methyl α -D-mannopyranoside. The supernatant fraction was removed and the resin was washed three times with 300 μ l of 0.1% (v/v) Triton X-100/500 mM NaCl/20 mM Tris/HCl (pH 7.4) containing 1 mM MgCl₂, 1 mM CaCl₂ and 1 mM MnCl₂. The bound fraction of protein was released by the addition of 40 μ l of 2 $\times$ Laemmli sample buffer containing 4% (w/v) SDS and then boiling for 4 min. If the sample was not heated to 100 °C, the release of glycoprotein from the resin was incomplete.

Inhibitor-affinity chromatography

Cell extracts (100 μ l) prepared in 1% (w/v) C₁₂E₈ were diluted 1:10 into 250 mM sodium citrate, pH 7.1, and added to 25 μ l of SITS-Affi-Gel [20] in the presence or absence of 1 mM 4,4'-diisothiocyanostilbene-2,2'-disulphonic acid (H₂DIDS) at 4 °C for 20 min. The supernatant was removed, the resin was washed with 0.1% (w/v) C₁₂E₈ in 228 mM sodium citrate, pH 7.1, and bound protein was eluted at room temperature with 75 μ l of Laemmli sample buffer containing 4% (w/v) SDS.

RESULTS

AE1 expressed in HEK cells contains a high-mannose oligosaccharide

Figure 2 shows an immunoblot of whole-cell extracts of HEK-293 cells expressing wild-type AE1 and mutant (N642D), in which the endogenous glycosylation site had been mutated, probed with an antibody directed against the C-terminus of human AE1. Vector-transfected cells showed no immunoreactive protein bands (Figure 2, lane 1). Cells transfected with a plasmid encoding wild-type AE1 contained an immunoreactive protein with a molecular mass of 95 kDa (Figure 2, lanes 2 and 5). The size of this band could be decreased by treatment of the cell extracts with either N-glycanase F (Figure 2, lane 6) or endo-

Figure 2 Immunoblot analysis of AE1 expressed in HEK-293 cells

HEK-293 cells were transfected with pcDNA3 vector or vector containing wild-type AE1, non-glycosylated mutant (N642D) or a mutant (K743N) containing a single N-glycosylation acceptor site at position 743. Cell lysates were treated with N-glycanase F (F) or endoglycosidase H (H) and compared with a mock-treated control sample (C). The samples were resolved on a 6% (w/v) polyacrylamide gel; AE1 was detected by immunoblotting. Symbols: ○, position of the non-glycosylated AE1; ●, position of N-glycosylated AE1. The positions of molecular mass markers are indicated (in kDa) at the left.

glycosidase H (Figure 2, lane 7). This showed that human AE1 expressed in HEK cells is N-glycosylated and that the oligosaccharide is of the high-mannose type.

The N642D mutant was expressed equally well in HEK cells, showing that N-glycosylation was not required for expression of AE1 in transfected cells (Figure 2, lanes 3 and 8). The protein had a lower molecular mass than that of wild-type AE1; its mobility was not shifted by treatment with either N-glycanase F (Figure 2, lane 8) or endoglycosidase H (Figure 2, lane 10). These results confirmed that this mutant did not contain any N-linked oligosaccharide.

The N-glycosylation status of AE1 was also assessed by lectin-affinity chromatography (Figure 3). HEK cell extracts in Triton X-100 were incubated with concanavalin A–Sephacrose in the absence or presence of methyl α -D-mannopyranoside. The unbound fraction and the bound fractions were isolated and the presence of AE1 in the fractions was determined by immuno-

Figure 3 Immunoblot showing the concanavalin A–Sephacrose binding properties of wild-type AE1 and the N642D and K743N mutants expressed in HEK-293 cells

Cell extracts were prepared by using 1% (v/v) Triton X-100 in lysis buffer and subjected to binding to concanavalin A–Sephacrose in the absence (–) and the presence (+) of 0.25 M methyl α -D-mannopyranoside. The unbound (U) and bound (B) fractions were resolved on a 10% (w/v) polyacrylamide gel; AE1 (indicated by arrow) was detected by immunoblotting. N-glycosylated mutants bind to concanavalin A–Sephacrose and appear in the bound (B) fraction in the absence (–) but not in the presence (+) of methyl α -D-mannopyranoside. Non-glycosylated mutants do not bind to concanavalin A–Sephacrose and appear only in the unbound (U) fractions.

Figure 4 Effect of folding mutations on the percentage of *in vitro* N-glycosylation at position 743 of the membrane-domain K743N mutant

Abbreviations: N642, wild-type membrane domain with the endogenous N-glycosylation site at Asn⁶⁴²; K743N, acceptor-site mutant; K743N-1, K743N, G713A, G714A triple mutant; K743N-2, K743N, A737P double mutant; K743N-3, K743N, T746I, P747A, G748A quintuple mutant. The results represent the averages of three independent translation reactions; error bars shown are the S.D.

blotting. Wild-type AE1 was able to bind to concanavalin A-Sepharose and this interaction was blocked by 0.25 M α -methylmannoside (Figure 3, lanes 1–4). In contrast, the N642D mutant failed to bind to the immobilized lectin (Figure 3, lanes 5–8). The results confirmed that human AE1 expressed in HEK cells contains a high-mannose oligosaccharide and that mutation of this single acceptor site prevented core N-glycosylation. Similar results were obtained by using transfected COS-7 cells (see Figure 7).

N-glycosylation at position 743

The hydrophilic intracellular (IC) loop 5 of human Band 3 can be cleaved at low yield at Lys⁷⁴³ by trypsin trapped inside resealed ghost membranes, suggesting a cytosolic localization for this region of AE1 [16]. A mutant (K743N) containing a new N-glycosylation acceptor site at this position was previously shown to be N-glycosylated at low efficiency during cell-free translation of the membrane domain, suggesting a luminal orientation for this loop during biosynthesis [15]. Similar results were obtained by cell-free translation of full-length AE1 (Table 1). However, the *in vitro* translation system might not report the correct topology of polytopic membrane proteins such as AE1 [27]. The K743N mutant was therefore expressed in HEK cells to determine the topology of this loop *in vivo*.

Figure 2 shows a comparison between the N-glycosylation status of the K743N mutant expressed in HEK cells and wild-type AE1 and the N642D mutant described above. The K743N mutant had the same mobility as deglycosylated AE1 and the N642D mutant (Figure 2, lane 4). Its mobility was not shifted by either N-glycanase F or endoglycosidase H (Figure 2, lanes 11–13). The K743N mutant also did not bind to the concanavalin A resin (Figure 3, lanes 9–12), confirming that it did not contain the high-mannose oligosaccharide. Expression of the K743N mutant in cells did not result in the production of an N-glycosylated protein. Either the K743N mutant was not N-

glycosylated in transfected cells or the glycosylated protein was rapidly degraded.

Protein segments that fold rapidly are poor substrates for the luminal oligosaccharyl transferase [28]. We propose that IC loop 5 is transiently exposed to the lumen of the endoplasmic reticulum during biosynthesis but normally folds rapidly, exposing Lys⁷⁴³ to the cytosol. To test this hypothesis, a series of different mutations were constructed in this loop, designed to disrupt its folding and increase the efficiency of N-glycosylation at Asn⁷⁴³. The glycosylation status of the mutants was determined by cell-free translation of the membrane domain. The sequence Gly⁷¹¹-Met-Gly-Gly within TM 9 has a high propensity for forming a reverse turn [6]. The double mutant G713A, G714A was designed to decrease the flexibility within this hydrophobic sequence proximal to the T-loop. The A737P mutant was designed to disrupt a region having a high helical propensity. The sequence Thr⁷⁴⁶-Pro-Gly-Ala is also predicted to form a reverse turn [6]. The mutant Thr⁷⁴⁶-Pro-Gly $\rightarrow$ Ile⁷⁴⁶-Ala-Ala was designed to disrupt this turn. Figure 4 shows that the levels of N-glycosylation at position 743 in the cell-free system of all three mutants were increased relative to the K743N mutant. These results show that this loop is accessible to the oligosaccharyl transferase in microsomes during cell-free translation and that mutations designed to disrupt folding of the loop resulted in an increase in the level of N-glycosylation at position 743.

N-glycosylation mutagenesis of extracytosolic loop 4

Previous translation studies *in vitro* on the membrane domain of AE1 showed that N-glycosylation acceptor sites in EC loop 4 had to be positioned a minimum distance of 12–14 residues from the adjacent TM segments to be N-glycosylated [15]. The N-glycosylation status of full-length AE1 containing mutations in EC loop 4 was compared by expression in a cell-free translation system and in transfected cells. Full-length AE1 and N-glycosylation mutants expressed in the cell-free system and in transfected cells often consisted of two closely spaced bands on SDS/PAGE. The upper band contained high-mannose oligosaccharide, whereas the lower band was not N-glycosylated because it was not sensitive to N-glycanase F or endoglycosidase H (results not shown). The addition of concanavalin A to the samples before gel electrophoresis specifically retarded the glycosylated form of AE1, enhancing the separation of the glycosylated and non-glycosylated bands.

Figure 5(A) shows an autoradiograph of the results obtained for the cell-free translation of full-length AE1 mutants. The percentage of N-glycosylation was determined from scans of the autoradiograph and integration of the areas corresponding to the upper glycosylated band and the lower non-glycosylated band. The results are summarized in Table 1. The maximum efficiency of N-glycosylation in this experiment was approx. 50%. Sites from positions 637 to 648 were N-glycosylated in agreement with previous results from the membrane domain [15]. An acceptor site at position 636 was poorly N-glycosylated, whereas acceptor sites at positions 634 and 635 were not N-glycosylated. The single site at position 633 was also N-glycosylated.

The same series of AE1 N-glycosylation mutants in EC loop 4 were expressed in HEK-293 cells to assess the extent of N-glycosylation. Figure 5(B) shows an immunoblot of transfected cells. The levels of expression of the different mutants in transfected cells were similar but varied from experiment to experiment depending on the efficiency of transfection. Several separate transfections were performed; the N-glycosylation results are summarized in Figure 5(C). A strict distance-de-

Figure 5 Scanning N-glycosylation mutagenesis of EC loop 4 determined *in vitro* (A) and *in vivo* (B, C)

(A) Autoradiograph showing the results of cell-free synthesis of N-glycosylation mutants. The N-glycosylation mutants were translated in rabbit reticulocyte lysate supplemented with microsomal membranes in the presence of [35 S]methionine. (B) Immunoblot of extracts of HEK-293 cells transfected with N-glycosylation mutants. Concanavalin A was added to the sample to increase the separation of the N-glycosylated protein from the non-glycosylated protein. Numbers indicate the position of the introduced novel acceptor sites into the N642D mutant. Symbols in (A) and (B): ○, position of the non-glycosylated AE1; ●, position of N-glycosylated AE1. (C) Quantification of the level of N-glycosylation of AE1 mutants in transfected HEK-293 cells from at least three separate transfections. Error bars indicate S.D. Abbreviation: wt, wild-type.

pendence for N-glycosylation of EC loop 4 was also observed *in vivo*. The first site distal to TM 7 to be N-glycosylated in cells was at position 633; the last site was at position 648. However, acceptor sites at positions 635 and 636, which were very poorly or not N-glycosylated *in vitro*, were N-glycosylated *in vivo*. Interestingly, an acceptor site at position 634 was not N-glycosylated, either in cells or in the cell-free system, although this acceptor site had an optimal sequence (Asn-Ser-Ser) for N-glycosylation [29]. Similar results were obtained in transfected COS-7 cells (results not shown) except that site 635 was poorly N-glycosylated in these cells. Overall, the *in vivo* and *in vitro* results were very similar, with the exception that sites 635 and

636 were more efficiently N-glycosylated in transfected HEK-293 cells.

Insertional scanning N-glycosylation mutagenesis

N-glycosylation acceptor sites in short extracytosolic loops in polytopic membrane proteins are not accessible to the luminal oligosaccharyl transferase [15,30]. To determine the topology of short loops in AE1, EC loop 4 bearing the endogenous N-glycosylation site at Asn⁶⁴² was inserted into the various loops (Figure 1) and their glycosylation status was assessed in trans-

Figure 6 Lectin gel-shift assay and immunoblot analysis of insertion mutants expressed in COS-7 cells

Extracts of transfected cells were resolved by SDS/PAGE on a gel that contained concanavalin A. AE1 was detected by immunoblotting. Numbers indicate the positions of insertions illustrated in Figure 1. Abbreviations: 428a, N642D EC loop 4 insert into EC loop 1 and mutated (N642D) endogenous site; 428b, N642D EC loop 4 insert into EC loop 1 and endogenous site; 428c, EC loop 4 insert into EC loop 1 and mutated (N642D) endogenous site. Symbols: ○, position of the non-glycosylated AE1; ●, position of N-glycosylated AE1. The open arrowhead indicates the position of the non-glycosylated insertion mutant; the filled arrowhead indicates the position of the glycosylated insertion mutant.

fectected COS-7 cells. A lectin gel-shift assay in which concanavalin A was incorporated into the upper portion of the separating gel was employed to distinguish between the N-glycosylated and

non-glycosylated forms of AE1. Four different insertion mutants into position 428 of EC loop 1 were created. These mutants contained no acceptor sites (mutant 428a), the endogenous site (mutant 428b), an acceptor site in the EC loop 1 insertion (mutant 428c) or a double N-glycosylation mutant (mutant 428d). The mobility of the insertion mutants on SDS/polyacrylamide gels was compared with that of the non-glycosylated 428a insertion mutant, which ran slightly more slowly than the N642D mutant (Figure 6; compare lanes 2 and 3). The lectin gel-shift assay showed that the endogenous site at Asn⁶⁴² in mutant 428b was N-glycosylated (Figure 6, lane 4), as was the insertion in EC loop 1 of the 428c mutant (Figure 6, lane 5). The double-insertion mutant showed a lower mobility than mutants 428b or 428c on normal SDS/polyacrylamide gels, which was consistent with N-glycosylation of the endogenous site and the insertion in EC loop 1 (results not shown). Insertions at positions 484, 754 and 854 were also efficiently N-glycosylated (Figure 6, lanes 6, 9 and 12). In contrast, the insertions into positions 731 and 785 were poorly N-glycosylated, which was inconsistent with a prominent extracellular localization for these regions of AE1. Insertions into positions 599 and 820, corresponding to IC loops 3 and 6, were not N-glycosylated (Figure 6, lanes 7 and 11), as expected for the intracellular location of these loops (Figure 1).

The N-glycosylation status of the insertion mutants was also determined by lectin-affinity chromatography (Figure 7). Extracts of transfected COS-7 cells were incubated with concanavalin A-Sepharose in the presence or absence of methyl α -D-mannopyranoside. As a control, normal AE1 and the N642D mutant were tested in the binding assay. In this series of experiments

Figure 7 Immunoblot analysis of concanavalin A-Sepharose binding properties of insertion mutants expressed in COS-7 cells

Transfected cells were lysed in 1% (v/v) Triton X-100 in lysis buffer and the extract was subjected to binding with concanavalin A-Sepharose in the absence (—) or presence (+) of 0.25 M methyl α -D-mannopyranoside. Equivalent volumes of the unbound (U) and bound (B) fractions were resolved on a 7.5% (w/v) polyacrylamide gel; AE1 was detected by immunoblotting. Mutants are designated as in the legend to Figure 6. N-glycosylated mutants bind to concanavalin A-Sepharose and appear in the bound (B) fraction in the absence (—) but not in the presence (+) of methyl α -D-mannopyranoside. Non-glycosylated mutants do not bind to concanavalin A-Sepharose and appear only in the unbound (U) fractions.

Figure 8 SITS-Affi-Gel binding assay of insertion mutants expressed in COS-7 cells

Upper panels: immunoblots of transfected cells were solubilized in 1% (w/v) C₁₂E₈ in lysis buffer and subjected to binding to SITS-Affi-Gel. Aliquots of the total sample (T), the fraction bound in the absence of H₂DIDS (B) and in the presence of H₂DIDS (D) were resolved on a 8% (w/v) polyacrylamide gel and AE1 was detected by immunoblotting. Abbreviation: wt, wild-type. Lower panel: quantification of binding assay shown in the upper panels. The open bars represent the percentage of the total AE1 present in the sample that bound to SITS-Affi-Gel in the absence of H₂DIDS (B). The stippled bars represent the percentage of the total AE1 present in the sample that bound to SITS-Affi-Gel in the presence of 1 mM H₂DIDS (D). Mutants are designated as in the legend to Figure 6. Properly folded mutants (K743N and insertion mutants 754, 820, 854) were able to bind to SITS-Affi-Gel at a similar level to the wild type. Other mutants exhibited a diminished binding similar to SAO Band 3.

approx. 60% of AE1 was bound by the resin, leaving the remainder in the unbound fraction (Figure 7, lanes 1 and 2). Larger amounts of AE1 in the sample could be bound by using higher amounts of resin; however, it became difficult to elute the bound protein. The AE1 bound to the resin was completely blocked by α -D-mannopyranoside (Figure 7; compare lanes 2 and 4). The control N642D mutant did not bind to the resin (Figure 7, lane 6). The control insertion mutant (428a) lacking N-

glycosylation sites did not bind to concanavalin A (Figure 7, lanes 5–8). The insertion mutant (428b), retaining the endogenous N-glycosylation site in EC loop 4 and containing a non-glycosylated insertion into EC loop 1, bound to the resin.

Figure 7 shows that mutants with insertion of EC loop 4 into positions 428, 484, 754, 785 and 854 bound to concanavalin A-Sepharose, whereas mutants with insertions into positions 599, 731 and 820 did not. N-glycosylation of insertions into

positions 428, 484, 754 and 854 and lack of N-glycosylation at 599 and 820 are consistent with the model shown in Figure 1. The insertion mutant at position 731 did not bind to the resin, suggesting that it was not N-glycosylated. This region of AE1 is not extracellular, as indicated in Figure 1, but is probably folded towards the cytosolic side of the membrane. The binding of insertion mutant 785 to concanavalin A-Sepharose shows that this mutant can be N-glycosylated in cells. However, the extent of N-glycosylated protein produced in cells varied greatly from experiment to experiment. Thus the disposition of this region of AE1 with respect to the membrane was difficult to assess.

Immunoprecipitation of AE1 labelled for 90 min with [35 S]-methionine in transfected COS cells confirmed the presence of N-glycosylated forms of insertion mutants 428, 484, 754 and 854 (results not shown). No N-glycosylated forms of mutants 599 or 820 were observed, whereas a low level of N-glycosylated forms of insertion at positions 731 and 785 were found. Interestingly, a pulse-chase experiment revealed that the N-glycosylated form of mutant 731 was unstable and was subsequently degraded, whereas the non-glycosylated form was stable (results not shown). The N-glycosylation status of the same set of AE1 insertion mutants was also assessed by cell-free translation (results not shown). The results of the experiments *in vivo* and *in vitro* are summarized in Table 1. The results were comparable except for insertion mutants at positions 731 and 785, which were N-glycosylated more efficiently in the cell-free system. A low level of N-glycosylation of the insertion mutant at position 820 was also observed *in vitro*. Overall, the results show that insertions at positions 428, 484, 754 and 854 were N-glycosylated and therefore would have an extracellular disposition.

Functional assessment of insertion mutants

Disruption of loops connecting the TM segments in AE1 might interfere with the folding of the polypeptide chain during biosynthesis and alter the native state of the mature protein. All of the insertion mutants were expressed at levels comparable to the that of wild-type protein. This suggests that a gross misfolding, which might result in the rapid degradation of the polypeptide chains, did not occur. To determine the native state of the insertion mutants an inhibitor-affinity chromatography technique was employed. SITS-Affi-Gel resin was developed as an affinity resin for the purification of anion exchangers [20,21]. An advantage of this technique is that the entire population of AE1 present within cells can be assessed. This is particularly important for AE1 expressed in transfected cells because the protein retains a high-mannose oligosaccharide, which suggests that the bulk of the protein might be retained in the endoplasmic reticulum.

COS-7 cells expressing AE1 mutants were extracted with $C_{12}E_8$ and incubated with SITS-Affi-Gel. Figure 8 (upper panels) is an immunoblot assay of the total and bound fractions in the absence and presence of H_2DIDS ; Figure 8 (lower panel) is a quantification of the immunoblot. AE1 expressed in cells bound to SITS-Affi-Gel and the binding could be blocked by 1 mM H_2DIDS (Figure 8, upper left panel, lanes 1–3). More than 75% of AE1 in the sample was bound to the resin in a DIDS-inhibitable manner. SAO Band 3 is a non-functional mutant that does not bind inhibitors [31–33]. The fraction of SAO AE1 that bound to the inhibitor column was greatly decreased compared with wild-type AE1 (Figure 8, upper left panel, lanes 4–6). This residual binding could be blocked with H_2DIDS , suggesting a weak interaction of SAO AE1 with immobilized SITS. The K743N mutant bound to the resin equally well as wild-type AE1 and this binding could be blocked with H_2DIDS (Figure 8, upper

right panel, lanes 1–3, and lower panel), showing that this point mutation does not affect inhibitor binding and is properly folded in cells.

Mutants containing insertions into EC loop 1 at position 428 exhibited a greatly decreased binding to SITS-Affi-Gel (Figure 8). This was independent of whether the inserted sequence was (mutant 428c) or was not (mutants 428a and 428b) N-glycosylated. The insertion into EC loop 1 did not change the overall topology of the protein because the endogenous site in EC loop 4 could still be N-glycosylated (mutant 428b). This suggests that insertion into EC loop 1 disrupted the inhibitor-binding site in AE1. The mutant containing the insertion at position 484 in EC loop 2 was N-glycosylated and also showed decreased binding to the affinity resin. The mutant with an insertion at position 731 in EC loop 5 was poorly N-glycosylated and had diminished binding to the resin. The mutant at position 785 in IC loop 6 was N-glycosylated to a variable extent and also had low binding. The insertions at position 754 in EC loop 6 and position 854 in EC loop 7, which were N-glycosylated, did not prevent binding. Interestingly, the mutant with an insertion at position 599 in IC loop 3, which was not N-glycosylated, did not bind to the resin. In contrast, the insertion mutant at position 820 in IC loop 6, which also was not N-glycosylated, bound to the resin as well as wild-type AE1. Thus the insertions at positions 754, 820 and 854 in EC 6, IC 6 and EC7 respectively did not disrupt the proper folding of AE1.

DISCUSSION

Expression of human AE1 in transfected cells

AE1 expressed in HEK-293 or COS-7 cells retains a high-mannose oligosaccharide. This greatly simplifies the analysis of N-glycosylation mutants. The lack of processing of the oligosaccharide chain to the complex type usually found on AE1 in human erythrocytes suggests that most of the protein might be retained in the endoplasmic reticulum in transfected cells. Murine AE1 expressed in HEK cells retains a high-mannose oligosaccharide and fails to exit from the endoplasmic reticulum [34]. This protein was functional because anion-exchange activity could be measured in isolated microsomal membrane vesicles prepared from these transfected cells. The lack of cell-surface expression in these experiments might, however, have been due to two spontaneous mutations in this particular mouse clone [35]. A significant fraction of human AE1 expressed in HEK cells is present at the cell surface because its transport function can be readily measured in intact cells [36]. AE1 might therefore be transported to the plasma membrane in transfected cells without oligosaccharide processing.

Requirements for N-glycosylation of loops in polytopic membrane proteins

In transfected HEK-293 and COS-7 cells, acceptor sites from positions 636 to 648 of EC loop 4 were N-glycosylated. In the cell-free system, acceptor sites from positions 637 to 648 were N-glycosylated, whereas sites 635 and 636 were poorly N-glycosylated (Table 1). In both systems the site at position 633 was also N-glycosylated, whereas the site at 634 was not. The difference between the results *in vivo* and *in vitro* are minor and might reflect more efficient N-glycosylation in cells or a subtle difference in the disposition of EC loop 4 in the two expression systems.

N-glycosylation of EC loop 4 in AE1 requires that acceptor sites be spaced a minimum distance from the ends of adjacent TM segments to reach the active site of the oligosaccharyl transferase. The N-glycosylated EC loop 4 in human AE1 is

bordered by two well-defined TM segments. TM7 contains a well-defined 21-residue hydrophobic sequence that ends at Ile⁶²⁴. Sites located at position 636 and beyond, 12 residues or more from the end of TM7, were N-glycosylated in cells. The hydrophobic domain of TM8 is predicted to begin at Phe⁶⁵⁹ or Pro⁶⁶⁰. The last N-glycosylation site in EC loop 4 was located at residue 648 both *in vivo* and *in vitro*, 11–12 residues from the beginning of TM8. Recent results with the use of scanning cysteine mutagenesis suggest that the hydrophobic domain of TM8 begins at Met⁶⁶⁴ and extends to Gln⁶⁸³ [36]. The last site of N-glycosylation in EC loop 4 is 16 residues from Met⁶⁶⁴. A more accurate determination of the distance between the end of TM segments and the sites of N-glycosylation will have to await a high-resolution crystal structure of AE1 that defines accurately the actual limits of the TM segments. The finding that sites at position 634 are not N-glycosylated suggests that the EC loop 4 sequence might be constrained at this point and that this acceptor site cannot fit properly into the active site of the oligosaccharyl transferase. An acceptor site at position 633 was N-glycosylated, showing that a site located only nine residues from the end of TM 7 is accessible to the transferase.

Functional consequences of insertions into loops

Insertions into some hydrophilic loops in AE1 resulted in a loss of inhibitor binding, suggesting that the insertion might have disrupted the proper folding of the protein. Similar findings were reported for the GLUT1 glucose transporter [18], the sodium/glucose co-transporter [27] and P-glycoprotein [37]. Some of the insertions in these proteins caused a loss of transport activity, whereas others had little or no effect on function. Insertions into EC1 or EC2 of AE1 produced a protein that did not bind to an inhibitor–affinity matrix. The insertion might cause the protein to misfold or the insertion might have directly occluded the inhibitor-binding site. Anion transport activity can be obtained in the plasma membrane of *Xenopus* oocytes by the co-expression of complementary fragments of AE1 divided into two within EC3 or EC4, but not with fragments divided into two within EC1 or EC2 [38,39]. Biosynthetic studies showed that the individual fragments divided in EC1 or EC 2 could associate with their complementary fragment [39]. Thus the integrity of the first two extracellular loops is essential for the proper functioning of AE1. Insertions at positions 731 or 785 also disrupted the inhibitor-binding site in the protein, suggesting that these regions are important for the proper folding of AE1.

An insertion into IC loop 3 also caused the protein to lose inhibitor binding, suggesting that this cytosolic loop has an important role in the AE1 structure. Interestingly, point mutations (R589S, R589C or R589H) in this loop cause distal renal tubular acidosis and an associated decrease in Band 3-mediated sulphate transport activity [40–42]. Chemical modification of Lys⁵⁹⁰ inhibits anion transport [43], suggesting that this region is important for transport function. It has, however, been reported recently [44] that co-expression of incompletely complementary fragments lacking entirely the segment encompassing TM6, TM7 and the intervening IC loop 3 could induce anion transport activity in *Xenopus* oocytes.

The insertion at position 820 in IC loop 6 did not affect inhibitor binding. Complementary fragments split in this intracellular loop could re-associate to form a functional transporter [45]. Thus the integrity of this loop is not required for stilbene disulphonate binding nor for anion transport. Insertion into the last extracellular loop at position 854 also did not eliminate inhibitor binding. This loop contains the epitope for a blood group antigen (Diego) owing to a P854L mutation [46].

This natural variant is functional, confirming that Pro⁸⁵⁴ is not required for transport. Our results suggest that a large insertion at this position does not impair the ability of the protein to fold properly or to bind inhibitors.

AE1 topology

The results indicate that the organization of TM segments in AE1 is largely consistent with the model presented in Figure 1. Insertions into EC loop 1 and EC loop 2 are N-glycosylated in a cell-free system [15] and in transfected cells. Lys⁴³⁰ is accessible to eosin maleimide [47] and reductive methylation [48] from the cell exterior. The extracellular disposition of EC loops 1 and 2 was also established by co-expression of complementary fragments [38] but remains in conflict with the results of Kalo, who used chemical labelling to localize Tyr⁴⁸⁶ to the cytosolic side of the membrane [49]. EC loop 3 is extracellular because it can be cleaved by treatment of intact erythrocytes with chymotrypsin [50], and the introduction of an N-glycosylation site in this loop results in N-glycosylation [15]. In other members of the AE gene family (trout AE1, AE2 and AE3) this loop contains a large insertion that is N-glycosylated, firmly establishing its extracellular disposition [51]. EC loop 4 contains the endogenous site of N-glycosylation and is therefore extracellular [1,2]. Introduced acceptor sites from positions 635 to 648 are N-glycosylated, showing that this entire region is extracellular.

The low level of N-glycosylation of the K743N mutant observed during cell-free translation suggests that this region of AE1 might have two different topologies in the cell-free system. In one topology this segment is exposed to the lumen of the endoplasmic reticulum and can become N-glycosylated. In the other topology this segment was exposed to the cytosolic side of the membrane. In transfected cells, only the latter topology is found, which is consistent with trypsin digestion experiments performed with the mature protein in the erythrocyte membrane [16]. It is possible that the protein with the alternative topology might have been degraded in transfected cells. Membrane proteins, such as P-glycoprotein, have been shown to exhibit alternative TM topologies [17,52]. This suggests that certain segments of polytopic membrane proteins have a dynamic aspect, especially during biosynthesis.

The insertion at position 731 was N-glycosylated in the cell-free system, suggesting that this insert must have been exposed transiently to the endoplasmic reticulum lumen during biosynthesis. Some N-glycosylation of the insertion at position 731 could be detected in cells; however, this aberrant form of the protein was rapidly degraded. The ability of TM9 to act as a topogenic signal anchor and that of TM 10 to act as a stop-transfer segment have been demonstrated in a cell-free system [53]. TM 9 might be flexible owing to the presence of three glycine residues in the middle of the hydrophobic sequence (Figure 1). The sequence Gly⁷¹¹-Met-Gly-Gly has a significant reverse-turn propensity [6]. A turn at this position would fold the T-loop containing Lys⁷⁴³ back towards the cytosol and would account for the lack of N-glycosylation of this residue. Mutations in these glycine residues results in increased N-glycosylation in mutant K743N of the membrane domain in the cell-free assay. Thus a disruption of the normal folding in this region displays a higher fraction of this loop to the endoplasmic reticulum lumen.

The insertion at position 754 was N-glycosylated in cells and in the cell-free system, placing EC 6 on the extracytosolic side of the membrane (Figure 1). Importantly, this insertion mutant bound to SITS-Affi-Gel, suggesting that it was properly folded. N-glycosylation of the insertion into the hydrophilic region at position 785 could be detected, indicating that this region can

also have access to the endoplasmic reticulum luminal oligosaccharyl transferase. However, this insertion mutant bound poorly to SITS-Affi-Gel, suggesting that the protein might not be properly folded. The large insertion could affect the local structure of the protein, which could compromise the veracity of the N-glycosylation assay. It is possible that the insertion at position 785 disrupted the stop-transfer function of the TM segment [53]. Another possibility is that this long hydrophobic segment is dynamic and can assume different positions in the membrane. Further work must be done to establish the topology of this region of AE1.

The insertion at position 820 is not N-glycosylated, which is consistent with the cytosolic disposition of this region [54]. The NMR solution structure of a peptide corresponding to IC6 (residues 796–841) indicates that it consists of two α -helices connected by a central proline-rich loop (Pro⁸¹⁵, Pro⁸¹⁶, Pro⁸²⁰) [55]. These terminal helices might lead directly into the adjoining TM helices. The insertion at position 854 is N-glycosylated and does not interfere with inhibitor binding. Mutation of Pro⁸⁵⁴ leads to the expression of a blood-group antigen, which is consistent with an extracellular disposition for this area [46].

The results presented in this paper show that the topology of AE1 expressed in transfected mammalian cells is similar to that determined by cell-free translation. The N-glycosylation of acceptor sites introduced into IC loop 5 observed *in vitro* probably reflects a transient exposure of this loop to the endoplasmic reticulum lumen during biosynthesis.

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