Processing of N-linked oligosaccharide depends on its location in the anion exchanger, AE1, membrane glycoprotein

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The human erythrocyte anion exchanger (AE)1 (Band 3) contains a single complex N-linked oligosaccharide that is attached to Asn⁶⁴² in the fourth extracellular loop of this polytopic membrane protein, while other isoforms (AE2, AE3 and trout AE1) are N-glycosylated on the preceding extracellular loop. Human AE1 expressed in transfected human embryonic kidney (HEK)-293 or COS-7 cells contained a high-mannose oligosaccharide. The lack of oligosaccharide processing was not due to retention of AE1 in the endoplasmic reticulum since biotinylation assays showed that approx. 30% of the protein was expressed at the cell surface. Moving the N-glycosylation site to the preceding extracellular loop in an AE1 glycosylation mutant (N555) resulted in processing of the oligosaccharide and production of a complex form of AE1. A double N-glycosylation mutant (N555/N642) contained both a high-mannose and a complex oligosaccharide chain. The complex form of the N555 mutant could be bio-

tinylated showing that this form of the glycoprotein was at the cell surface. Pulse-chase experiments showed that the N555 mutant was efficiently converted from a high-mannose to a complex oligosaccharide with a half-time of approx. 4 h, which reflected the time course of trafficking of AE1 from the endoplasmic reticulum to the plasma membrane. The turnover of the complex form of the N555 mutant occurred with a half-life of approx. 15 h. The results show that the oligosaccharide attached to the endogenous site in extracellular loop 4 in human AE1 is not processed in HEK-293 or COS-7 cells, while the oligosaccharide attached to the preceding loop is converted into the complex form.

Key words: Band 3, endoplasmic reticulum, glycosylation, trafficking, turnover.

INTRODUCTION

The human erythrocyte anion exchanger (AE)1 (Band 3) contains a single site of N-glycosylation at Asn⁶⁴² in the fourth extracellular loop of the protein [1–3]. This oligosaccharide consists of either an elongated polylactosaminyl structure [4] or a shorter, more typical, complex structure [5] resulting in two Band 3 glycoprotein populations [6]. In the human disease, hereditary erythroblastic multinuclearity with a positive acidified-serum lysis test (HEMPAS), the oligosaccharide on red cell glycoproteins is not completely processed but retains a high-mannose structure due to a defect in mannosidase II [7–10]. The anion transport activity of HEMPAS erythrocytes is normal showing that the high-mannose form of AE1 is fully functional [9]. Interestingly, a mannosidase II deficient mouse lacked complex N-glycans on its erythrocyte glycoproteins but contained complex glycoproteins in non-erythroid cells, resulting in a HEMPAS phenotype [11]. This suggested that an alternative oligosaccharide processing pathway not involving mannosidase II is available in cells other than those of the erythroid lineage.

Expression of human AE1 in transfected human embryonic kidney (HEK)-293 or COS-7 cells results in the production of a protein that retains a high-mannose oligosaccharide [12]. A similar observation was made previously for mouse AE1 [13]. Human AE1 is functionally expressed at the cell surface of transfected cells [14]. These results suggest that the lack of oligosaccharide processing is not entirely due to retention of the glycoprotein in the endoplasmic reticulum and that a high-mannose form of AE1 is functional. In cell-free translation studies of the topology of AE1 we found that a novel N-

glycosylation acceptor site introduced at Asn⁵⁵⁵ in extracellular loop 3 can be N-glycosylated [15]. In the present study we report that expression of this AE1 mutant (N555) in transfected HEK-293 or COS-7 cells resulted in the production of a glycoprotein containing a complex oligosaccharide. The processing of the oligosaccharide attached to AE1 is therefore dependent on the location of the oligosaccharide in this polytopic membrane protein. The processing of the oligosaccharide on the N555 mutant allowed the study of the trafficking of human AE1 in transfected cells from its site of synthesis, in the endoplasmic reticulum, to its final destination, in the plasma membrane.

EXPERIMENTAL

Materials

Materials were purchased from the following suppliers (in parentheses): pcDNA3 (Invitrogen, San Diego, CA, U.S.A.); TransformerTM site-directed mutagenesis system (Clontech Laboratories, Palo Alto, CA, U.S.A.); T7 DNA sequencing kit, Protein G-Sepharose and restriction enzymes (Amersham Pharmacia Biotech, Baie d'Urfé, Quebec, Canada); Dulbecco's modified Eagle's medium (DMEM), calf serum, penicillin and streptomycin (Gibco BRL, Burlington, Ontario, Canada); [³⁵S] methionine, consisting of approx. 73% [³⁵S] methionine and 27% [³⁵S]cysteine (Mandel Scientific, Guelph, Ontario, Canada); endoglycosidase H, N-glycanase F and goat peroxidase-conjugated anti-rabbit IgG (New England Biolabs, Mississauga, Ontario, Canada); octa(ethylene glycol) dodecyl ether (C₁₂E₈; Nikko Chemical Co., Tokyo, Japan); chemiluminescence kit

Abbreviations used: AE, anion exchanger; C₁₂E₈, octa(ethylene glycol) dodecyl ether; HEK, human embryonic kidney; HEMPAS, hereditary erythroblastic multinuclearity, with a positive acidified-serum lysis test; NHS-SS-biotin, sulphydryl-succinimidyl 2-(biotin-amido)ethyl-1,3-dithiopropionate.

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(Boehringer Mannheim, Laval, Quebec, Canada); ImmunoPure® immobilized streptavidin and EZ-link[™] sulphy-succinimidyl 2-(biotin-amido)ethyl-1,3-dithiopropionate (NHS-SS-biotin; MJS BioLynx Inc., Brockville, Ontario, Canada).

Mutagenesis and DNA sequencing

The entire coding sequence for human AE1 was inserted into the *Xho*I and *Bam*HI sites of pcDNA3 [12]. The following AE1 N-glycosylation mutants were constructed as described previously [15]: N642D, a non-glycosylated mutant with a mutated endogenous site; N555, a mutant with a single novel site at Asn⁵⁵⁵ (Y555N/V557T) and a mutated (N642D) endogenous site; and N555/N642, a double N-glycosylation mutant with a novel site at Asn⁵⁵⁵ (Y555N/V557T) and the endogenous site.

Expression of AE1 in transfected cells

HEK-293 or COS-7 cells were transfected with 5 µg of plasmid DNA per 10 cm diameter plate and grown in DMEM supplemented with 10% (v/v) calf serum, 0.5% penicillin and 0.5% streptomycin under air/CO₂ (19:1) at 37 °C, as described previously [12]. Cell extracts were prepared by detergent solubilization of the cells 48 h after transfection, as described below.

SDS/PAGE and immunoblotting

Proteins were resolved by SDS/PAGE [16]. AE1 was detected by immunoblotting using a rabbit polyclonal antibody directed against the synthetic peptide corresponding to the C-terminal 16 residues of human AE1 [12].

Enzymic deglycosylation and lectin binding

Cell extracts (50 µl) prepared from transfected cells using 1% (v/v) C₁₂E₈ were incubated for 1 h at 37 °C with no additions, 1000 units (1 µl) of endoglycosidase H (1 000 000 units/ml) or 500 units (1 µl) of N-glycanase F (500 000 units/ml), followed by addition of 1 vol. of 2 × Laemmli sample buffer containing 4% (w/v) SDS.

The binding of AE1 mutants to immobilized concanavalin A was determined as described previously [12]. Briefly, cell extracts (50 µl) in 1% (v/v) C₁₂E₈ were incubated with 25 µl of concanavalin A-Sepharose in the absence and presence of 0.25 M methyl α-D-mannopyranoside. The supernatant represented the unbound fraction while the bound fraction was released by boiling the resin in 2 × Laemmli sample buffer. A similar binding assay was performed using wheat germ agglutinin-Sepharose in the presence and absence of 0.3 M N-acetyl-D-glucosamine.

Cell surface biotinylation

HEK-293 cells were grown in 6-well culture dishes for 48 h after transfection. The cells were washed once with ice-cold borate buffer (10 mM boric acid, 154 mM NaCl, 7.2 mM KCl and 1.8 mM CaCl₂, pH 9.0). Cells were subsequently treated with 1 ml of 0.8 mM EZ-Link[™] NHS-SS-biotin in borate buffer for 15 min at 0 °C. Parallel control cells were not treated with the biotinylation reagent. Cells were washed with 0.192 M glycine/25 mM Tris, pH 8.3 to quench the reaction. The cells were lysed in RIPA buffer [10 mM Tris/HCl (pH 7.5), 1% (w/v) deoxycholate, 1% (v/v) Triton X-100, 0.1% SDS, 150 mM NaCl and 1 mM EDTA] containing protease inhibitors (200 µM PMSF, 2.8 µM E64, 1 µM leupeptin and 1 µM pepstatin), followed by centrifugation at 14 000 g for 15 min to remove insoluble material. An aliquot of the lysate was saved for

measurement of the total AE1 content. Immunopure immobilized streptavidin resin (100 µl) was added to the lysate and incubated for 1 h at 4 °C to bind biotinylated protein. Addition of a larger volume of beads did not increase the yield of bound protein. As an additional control for non-specific binding, samples were also incubated with Sepharose beads lacking streptavidin. The supernatant was removed and an aliquot saved for measurement of AE1 in the unbound fraction. The beads were washed with RIPA buffer and bound protein was eluted by addition of Laemmli sample buffer containing 5% (v/v) 2-mercaptoethanol for 1 h at room temperature. The content of AE1 in the total, unbound and bound fractions was determined by immunoblotting.

Pulse-chase experiments

HEK-293 or COS-7 cells were grown at 37 °C in 35 mm diameter dishes for 24 h, after transfection with 0.6 µg of plasmid DNA. The cells were incubated for 45 min in methionine- and cysteine-free DMEM without calf serum. Cells were labelled with 100 µCi/ml [³⁵S]methionine for 10 min. The radioactive medium was removed and the cells were rinsed once with PBS. The cells were then chased for up to 24 h by incubation in DMEM containing 10% calf serum, and 10 µg/ml cycloheximide to inhibit further protein synthesis. For turnover studies the cells were labelled for 90 min with 20 µCi/ml [³⁵S]methionine and then chased without cycloheximide for the indicated times up to 48 h. Cells were harvested at timed intervals, washed with PBS and then solubilized in 1 ml of RIPA buffer. AE1 was immunoprecipitated using 4 µl of antibody serum directed against the C-terminal of human AE1, followed by capture on 40 µl of Protein G-Sepharose beads. The proteins were eluted from the resin using 50 µl of 2 × SDS sample buffer at room temperature for 10 min. Proteins were resolved by SDS/PAGE and were detected by autoradiography.

RESULTS

AE1 N-glycosylation mutants

Human AE1 consists of 12 transmembrane segments with a single site of N-glycosylation at Asn⁶⁴² (Figure 1A). Three mutants of AE1 (N642) were constructed that contained no N-glycosylation acceptor sites (N642D), a single novel site at Asn⁵⁵⁵ (N555) or a double N-glycosylation mutant with a novel site at Asn⁵⁵⁵ and the endogenous site at Asn⁶⁴² (N555/N642) [15,17]. Figure 1(B) shows an immunoblot of HEK-293 cells transfected with AE1 constructs N642, N642D, N555 or N555/N642. Wild-type AE1 (lane 2) was present in transfected cells as a single band with a slightly higher molecular mass than the N642D mutant (lane 1) due to the presence of a high-mannose N-linked oligosaccharide [12]. In contrast, the N555 mutant (lane 3) was present as two closely spaced bands. The lower band was at the same position as wild-type high-mannose protein, while the second band had a higher molecular mass. These results suggest that a fraction of the N555 mutant had been processed to a high molecular mass form containing a complex oligosaccharide. Scans of the immunoblots showed that approximately one-third of the N555 mutant was present as the higher molecular mass form. The N555/N642 mutant (lane 4), with two N-glycosylation sites, also ran as two bands but with even higher molecular masses than the N555 mutant, indicating that both sites were N-glycosylated.

Enzymic deglycosylation and lectin binding

Enzymic deglycosylation was performed in order to characterize the nature of the oligosaccharide attached to the various AE1

Figure 1 N-glycosylation mutants of human AE1

(A) Folding model of human AE1 showing the location of Asn⁵⁵⁵ and Asn⁶⁴². (B) Immunoblot of extracts of HEK-293 cells transfected with AE1 N-glycosylation mutants. HEK-293 cells were transfected with pcDNA3 vector containing the non-glycosylated mutant (N642D), wild-type AE1 (N642), the N555 mutant with a single acceptor site at position 555 (N555) or the N555/N642 mutant with two acceptor sites at positions 555 and 642 (N555/N642). Cell extracts were prepared by solubilizing cells with 1% (v/v) C₁₂E₈, 150 mM NaCl and 5 mM sodium phosphate, pH 7.4 (PBS). Proteins were resolved by SDS/PAGE using an 8% (w/v) polyacrylamide gel, transferred to nitrocellulose and AE1 was detected by immunoblotting. Ct, C-terminus; Nt, N-terminus.

mutants (Figure 2). Wild-type AE1 was sensitive to endoglycosidase H treatment (Figure 2, lanes 1–3) consistent with a high-mannose structure [12]. The lower band of the N555 mutant was also sensitive to this enzyme (Figure 2, lanes 4–6), showing that this form of the protein contained a high-mannose oligosaccharide. The upper band, in contrast, was resistant to this enzyme treatment, but its mobility could be shifted by N-glycanase F. This suggests that the upper band contained a complex oligosaccharide. In the double mutant, the lower and upper bands were both sensitive to endoglycosidase H (Figure 2, lanes 7–9), suggesting that each contained a high-mannose oligosaccharide. The lower band shifted down to the position of the non-glycosylated AE1 showing that it contained two high-mannose oligosaccharides. In contrast, the upper band was shifted down to the position of the non-glycosylated AE1 only after treatment with N-glycanase F. This showed that the upper band contained both high-mannose and complex oligosaccharides. There was no evidence of a higher molecular mass form of this mutant that contained two complex oligosaccharide chains. The results are consistent with the oligosaccharide at position 555 being high-mannose and the oligosaccharide at

Figure 2 Immunoblot showing enzymic deglycosylation of N642, N555 or N555/N642

Cell extracts were incubated without enzyme addition (C), with endoglycosidase H (H), or with N-glycanase F (F). AE1 was detected as in Figure 1. Open circle, non-glycosylated AE1; closed circle, high-mannose form of AE1; asterisk, complex form of N555 mutant; double closed circle, double high-mannose form of N555/N642 mutant; asterisk/closed circle, high-mannose and complex form of N555/N642 mutant.

Figure 3 Immunoblot showing the concanavalin A-Sepharose binding properties of N642D, N642, N555 and N555/N642

Cell extracts of transfected HEK-293 cells were prepared by using 1% (v/v) C₁₂E₈ in lysis buffer and subjected to incubation with concanavalin A-Sepharose in the absence (—) and presence (+) of 0.25 M methyl α -D-mannopyranoside. The unbound (U) and bound (B) fractions were resolved by SDS/PAGE and AE1 was detected by immunoblotting.

position 642 of the same molecule being processed to a complex oligosaccharide.

Oligosaccharide chains must be accessible to the processing enzymes in the Golgi in order to be converted to a complex-type oligosaccharide [18,19]. The accessibility of the oligosaccharide chains at positions Asn⁵⁵⁵ and Asn⁶⁴² of AE1 was compared by digestion of the solubilized mutant proteins with various concentrations of N-glycanase F. It was found that the oligosaccharide at position 555 was 10-fold more sensitive to enzymic deglycosylation than the site at position 642 (results not shown). This result indicates that the oligosaccharide at position 555 may also be more accessible to modification by the processing enzymes in the secretory pathway.

Lectin affinity chromatography was performed to confirm the nature of the oligosaccharide in the AE1 mutants (Figure 3). AE1 expressed in HEK-293 cells bound to concanavalin A-Sepharose (Figure 3, lanes 5–8), while the non-glycosylated N642D mutant did not (Figure 3, lanes 1–4). The binding of AE1 to concanavalin A could be blocked by methyl α -D-mannopyranoside (Figure 3, lane 7), confirming the specificity of the interaction with this lectin. In this particular experiment approximately half of the AE1 was bound to the lectin. All of the AE1 could be bound by using higher amounts of resin, however, the

Figure 4 Immunoblot analysis of cell surface biotinylation of HEK-293 cells expressing N642 (left panel), N642D (middle panel) or N555 (right panel)

Cells extracts were prepared from biotinylated cells and incubated with streptavidin–Sepharose. The total sample (T), unbound fraction (U) and bound fraction (B) were resolved by SDS/PAGE and AE1 was detected by immunoblotting. AE1 (N642) and the N642D mutant each ran as predominantly single monomeric protein bands. The high molecular mass bands near the top of the gel represent undissociated AE1 dimers. The N555 mutant ran as two closely spaced bands. The upper band contained complex oligosaccharide while the lower band contained high-mannose oligosaccharide. The bound fraction was overloaded 12× with respect to the total sample and the unbound fraction (i.e. the supernatant) to get comparable band densities.

protein became more difficult to elute from the lectin. The ability of wild-type AE1 to bind to concanavalin A is consistent with the presence of a high-mannose oligosaccharide. The lower band of the N555 mutant also bound to this lectin while the upper band did not (Figure 3, lanes 9–12). This is consistent with the deglycosylation results, which indicated that the upper band contained a complex oligosaccharide while the lower band retained a high-mannose oligosaccharide structure. The lower band of the double mutant was bound completely to this resin, whereas the upper band bound poorly (Figure 3, lanes 13–16). The yield of protein in the bound fraction was very low and may have been due to the high avidity of the double N-glycosylated high-mannose protein for the resin. We were surprised to find that the upper band of the double mutant bound poorly to the concanavalin A resin. This suggests that the protein has a lower avidity for the resin compared with the high-mannose protein or that the presence of the large complex chain in the neighbouring loop compromised the binding of the high-mannose chain. The upper band of the N555 mutant bound to immobilized wheat-germ agglutinin while the lower band did not (results not shown) indicating that the upper band did contain complex oligosaccharide. Thus the N555 mutant is also present in two forms in transfected cells, a lower molecular mass form containing a high-mannose oligosaccharide and a higher molecular mass form containing a complex oligosaccharide.

Cell surface biotinylation

AE1 expressed in HEK-293 cells contained a high-mannose oligosaccharide that was not processed to a complex oligosaccharide. This may be due to retention of the protein in the endoplasmic reticulum or to inefficient oligosaccharide processing as AE1 moves through the secretory pathway. Cell surface biotinylation was used to determine the level of cell surface expression of the AE1 mutants. Cells were treated with NHS-SS-biotin and the fraction of AE1 that was biotinylated was determined by binding to streptavidin beads. The total, unbound and bound fractions were analysed for AE1 by immunoblotting (Figure 4). AE1 could be biotinylated by treating intact transfected cells with the non-penetrating biotinylation reagent (Figure 4, left panel). The bound fraction of AE1 was sensitive to endoglycosidase H (results not shown). The level of cell surface expression of AE1 in HEK-293 cells, determined from the bound

Figure 5 Pulse–chase experiments using COS-7 cells transfected with the N555 glycoform

Transfected cells were labelled with [³⁵S]methionine for 10 min followed by a chase for the indicated times. At each time point cells were lysed with RIPA buffer and AE1 was immunoprecipitated and samples were resolved by SDS/PAGE. The upper panel is an autoradiograph showing the time-dependent conversion of the high-mannose form (open circle) to the complex form (closed circle). The lower panel is a plot of the percentage of the complex oligosaccharide relative to the total oligosaccharide (complex plus high-mannose).

fraction, was only 10%. The amount in AE1 remaining in the unbound fraction relative to the total, however, indicated a decrease of 30%. This suggests that release of the biotinylated protein from the beads was not very quantitative. No AE1 bound to the resin without prior treatment of the cells with the biotinylation reagent (results not shown) and no AE1 binding to Sepharose beads lacking streptavidin was detected, confirming the specificity of the labelling. The results suggest that a significant fraction (30%) of human AE1 had moved through the secretory pathway to the plasma membrane without extensive modification to its oligosaccharide. The presence of AE1 at the cell surface is in agreement with functional assays that can measure $\text{Cl}^-/\text{HCO}_3^-$ exchange in intact cells [14] and similar biotinylation assays [20].

The N642D mutant could also be biotinylated, similarly to wild-type AE1 (Figure 4, middle panel). The amount of AE1 in the unbound fraction indicated that approx. 30% of the N642D mutant was present at the cell surface. This result suggests that N-glycosylation of AE1 is not a necessary requirement for cell-surface expression, in agreement with previous studies using AE1 expression in *Xenopus* oocytes [21].

Cell surface biotinylation assays of the cells transfected with the N555 mutant showed that the upper band could be quantitatively biotinylated while the lower band could not (Figure 4, right panel). The upper band consists of a glycoprotein that has moved through the secretory pathway and whose oligosaccharide was processed from high-mannose to complex. The lower band is AE1 that is located in the endoplasmic reticulum and contains a high-mannose oligosaccharide. Based on the decreased amount of

Figure 6 Turnover of the N555 mutant in transfected COS-7 cells

Transfected cells were labelled with [35 S]methionine for 90 min followed by a chase for the indicated times, as described in Figure 5. The upper panel is an autoradiograph showing the time-dependent loss of the high-mannose form (open circle) and the complex form (closed circle). The lower panel is a plot of the percentage of radiolabelled high-mannose and complex AE1 remaining, relative to the amount at the end of the labelling period.

AE1 in the unbound fraction, the level of cell surface expression of the N555 mutant was similar (30 %) to that of wild-type AE1.

Time course of oligosaccharide processing

The biosynthetic relationship between the high-mannose and complex forms of the N555 mutant was examined by pulse–chase experiments. Figure 5 (upper panel) shows an immunoprecipitate of a whole-cell detergent lysate of transfected COS-7 cells that were labelled with [35 S]methionine for 10 min and chased for the indicated intervals. The N555 mutant was initially synthesized as a lower molecular mass band that decreased in amount during the chase period with the concomitant appearance of a higher molecular mass form. The lower molecular mass form was sensitive to endoglycosidase H while the upper band was resistant (results not shown). The data indicate that there is a time-dependent conversion of a high-mannose form of the N555 mutant into a complex oligosaccharide form, with a half-time of approx. 4 h (Figure 5, lower panel). This conversion reflects the transit time of the glycoprotein from its site of synthesis in the endoplasmic reticulum to the plasma membrane. Greater than 75 % of AE1 was converted from a high-mannose form into complex form over a 21 h period. This high percentage indicates that there was nearly complete conversion of the high-mannose oligosaccharide into the complex form, and that very little degradation of the high-mannose form in the ER must have occurred over the time period. Similar pulse–chase experiments with wild-type AE1 showed that the protein ran as a single band and that there was no conversion to a higher molecular mass form (results not shown). This band remained sensitive to endoglycosidase H indicating that it contained high-mannose oligosaccharide.

Stability of N-glycosylation mutants

In order to determine the stability of the N555 mutant, cells were labelled with [35 S]methionine for 90 min and then chased for up to 45 h (Figure 6). The amount of the complex form of the N555 mutant initially increased due to continued conversion from the high-mannose form and then decreased with a half-life of approx. 15 h. This long half-life indicates that the mature form of human AE1 is quite stable in the plasma membrane of transfected cells. The high-mannose form of the N555 mutant also decreased during the chase period. In this case, the amount of radiolabelled high-mannose form present reflected a complex balance between conversion into the complex form, degradation within the ER and residual protein synthesis that may have occurred during the chase period.

DISCUSSION

N-glycosylation of anion exchangers

AE1 is a member of a gene family of anion exchangers responsible for the electroneutral exchange of chloride and bicarbonate across cell membranes. Mammalian AE1 proteins all contain a single site of N-glycosylation in the fourth extracellular loop, whereas AE2, AE3 and trout AE1 contain N-glycosylation sites in the preceding extracellular loop. In the present study we have shown that oligosaccharide attached to the fourth extracellular loop of human AE1 is retained as a high-mannose oligosaccharide in transfected cells. Mutants in which an N-glycosylation site is engineered into the third extracellular loop were processed to complex oligosaccharides at this site. Thus the processing of the oligosaccharide on human AE1 is dependent on its location. Murine AE2, which contains N-glycosylation sites on the third extracellular loop, was processed to a complex oligosaccharide in transfected cells [13]. This site-dependence of N-glycosylation processing has also been observed for secreted proteins that contain high-mannose and complex oligosaccharides at different sites in the same protein [19].

The results presented in this paper show that transfected HEK-293 and COS cells are unable to efficiently process the oligosaccharide on human AE1, although the protein can move through the secretory pathway to the plasma membrane. In contrast, when the oligosaccharide is attached to Asn⁵⁵⁵ in the third loop it is readily processed to the complex form by both of these cell types. In a double N555/N642 mutant the results indicate that the oligosaccharide chain at Asn⁵⁵⁵ was processed while the oligosaccharide chain at Asn⁶⁴² was not. This result shows that although AE1 had moved through the secretory pathway to the plasma membrane, the site at Asn⁶⁴² was refractory to modification to the complex form. An important feature of oligosaccharide processing is that cells vary in their ability to process certain glycoproteins [18]. Human AE1 is normally processed to a complex oligosaccharide during red cell development [4]. In contrast, neither HEK-293 nor COS-7 cells were capable of efficiently processing the oligosaccharide chain of AE1 at Asn⁶⁴². HEK-293 cells are clearly not deficient in oligosaccharide processing enzymes, since the oligosaccharide at Asn⁵⁵⁵ in AE1 can be converted into a complex oligosaccharide.

The ability of an oligosaccharide chain to be processed depends on the degree of accessibility of the site to the processing enzymes in the Golgi [18,19]. We found that the oligosaccharide on extracellular loop 3 is more sensitive to cleavage by N-glycanase F than the oligosaccharide on loop 4. Extracellular loop 3 in human AE1 is also accessible to cleavage by various proteases [22,23]. Moving extracellular loop 3 to other positions in AE1 also resulted in production of a high-mannose glycoprotein in

transfected cells [12]. This suggests that it is the structure of loop 3 rather than its position in the protein that restricts processing. The oligosaccharide at Asn⁶⁴² on AE1 may be a poor substrate for the processing pathway present in the transfected cells.

In the human disease HEMPAS, red cell glycoproteins retain their high-mannose oligosaccharide due to a deficiency in mannosidase II [8,10]. A mannosidase II knock-out mouse is unable to process N-linked oligosaccharide present on erythrocyte glycoproteins, but can still process glycoproteins normally in other cells [11]. This suggests that mannosidase II is essential for the processing of oligosaccharide chains on glycoproteins in the erythroid lineage. In other cells an alternative pathway is proposed which bypasses the mannosidase II step. The results suggest that mannosidase II in transfected cells may not be able to process the oligosaccharide attached to Asn⁶⁴². AE1 in transfected HEK-293 cells contains only high-mannose oligosaccharide and therefore resembles AE1 in HEMPAS red cells.

AE1 trafficking to the plasma membrane

The nearly complete conversion of the high-mannose form of the N555 mutant to the complex form shows that the intracellular fraction of AE1 was fully competent for trafficking to the plasma membrane. These findings are in contrast with cystic fibrosis conductance transmembrane regulator ('CFTR'), in which only a fraction of the protein moved to the plasma membrane [24,25]. The remainder was retained in the endoplasmic reticulum and degraded by a proteasome-mediated pathway [26]. The complex form of the N555 mutant present in the plasma membrane had a turnover of 15 h, showing that the mature AE1 was relatively stable in transfected cells.

The N555 mutant moved from its site of synthesis in the endoplasmic reticulum to the plasma membrane with a half-time of 4 h in transfected cells. This transit time is similar to the 2 h half-time observed for murine AE2 in transfected cells [13]. Pulse-chase experiments in murine spleen cells showed that AE1 became endoglycosidase H resistant with a faster half-time of 30 min [27]. Transfected cells, unlike pre-erythroid cells, may not contain all the specific proteins required for the efficient assembly of AE1 [13]. AE1 in HEK-293 cells interacts transiently with the chaperone calnexin, which may be involved in facilitating its folding and exit from the endoplasmic reticulum [28]. Evidence suggests that other proteins present in the red cell, such as ankyrin, glycophorin A, and Band 4.2, may be required for the assembly of AE1 in the plasma membrane. Ankyrin has been reported [29] to associate with AE1 in a pre-Golgi compartment in transfected cells, which may facilitate the exit of AE1 from the endoplasmic reticulum. The trafficking of AE1 to the plasma membrane in *Xenopus* oocytes is enhanced by co-expression of glycophorin A [30] while Band 3 from glycophorin-deficient red cells has an increased level of N-glycosylation, suggesting a reduced transit time through the Golgi [31,32]. Human erythroleukemia K562 cells express endogenous glycophorin A and transfection of these cells with AE1 using a retroviral system results in functional cell surface expression of AE1 [33]. Erythrocytes from AE1 knock-out mice are devoid of Band 4.2 [34] and glycophorin A [35], suggesting that both Band 4.2 and glycophorin A form a complex with AE1.

AE1 exists in mature red cells as a mixture of dimers and tetramers, the tetramers providing the binding site for ankyrin [36,37]. Ankyrin-deficient red cells lack Band 3 tetramers, suggesting that ankyrin is required for AE1 tetramer formation [38]. Murine AE1 forms dimers in transfected cells [39] and the protein is functional in the endoplasmic reticulum [13]. As HEK-293 cells are deficient in ankyrin, tetramer formation was not

expected. In mouse erythroblasts AE1 tetramers are formed in early erythroblasts, are found in the microsomal fraction and are subsequently incorporated into the plasma membrane [40]. Dimers present at the late stages of erythroid maturation move rapidly to the plasma membrane and can recycle to the microsomal fractions. In chicken erythroblasts AE1 initially moves to the plasma membrane in a high-mannose form and is then internalized and recycled to the Golgi, where AE1 associates with ankyrin and the oligosaccharide is processed to a complex form [41]. The AE1-ankyrin complex then moves to the plasma membrane where the complex links to spectrin. The first stage is similar to what was observed in the present study, where the oligosaccharide on human AE1 escaped modification during the initial transit of the protein through the secretory pathway to the plasma membrane. Tetramer formation and ankyrin binding may be required for oligosaccharide processing of human AE1, which may occur during subsequent recycling of the protein from the plasma membrane to the Golgi. Tetramer formation may make human AE1 a substrate for the processing enzymes, or perhaps tetrameric AE1 follows a different processing route back to the plasma membrane. The processing of the oligosaccharide on human AE1 in transfected cells depends on the location of the oligosaccharide chain, showing that these cells contain the required processing enzymes, but that the oligosaccharide at Asn⁶⁴² in AE1 is refractory to them.

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