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FULL PAPER

Cell surface expression and internalization of the murine erythroid AE1 anion exchanger tagged with an extracellular FLAG epitope

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Abstract

Anion exchanger 1 (AE1) is the most abundant integral membrane protein in red cells and is essential for maintaining red cell mechanical stability. However, the mechanism for the assembly of AE1 into the membrane skeletal network remains unknown. Several mutants of murine AE1 tagged with an N-terminal enhanced green fluorescent protein (EGFP) and/or an extracellular FLAG epitope inserted adjacent to the *N*-glycosylation site were prepared, and their expression was analyzed in HEK293 or COS-1 cells by immunofluorescence microscopy, biotinylation, and deglycosylation. The EGFP- and FLAG-tagged AE1 mutant, as well as the wild-type AE1, exhibited cell surface expression in transfected cells and showed a rapid internalization that appeared to occur through the early endosome into the Golgi apparatus. Interestingly, the form of the protein with an endoglycosidase H (endo H)-sensitive *N*-glycan was the major component of EGFP-tagged and wild-type AE1. By contrast, the polypeptide with an endo H-resistant oligosaccharide was the predominant form of FLAG-tagged AE1. These data demonstrate that the processing of N-glycan is not a prerequisite to cell surface expression of AE1 and suggest that the FLAG tag insertion altered the accessibility of the *N*-glycan to enzymes in the Golgi which facilitate processing of oligosaccharides. Although whether this structural alteration would affect the structural and functional properties of AE1 remains unknown, cell surface expression and endocytic internalization of FLAG-tagged AE1 mutants indicate that these mutants are suitable for studying the mechanisms of the assembly and plasma membrane insertion of AE1.

Keywords: anion exchanger 1 (AE1), erythroid, FLAG, membrane skeleton, red cell membranes

Introduction

Anion exchanger 1 (AE1, also called band 3 in red cells) is the most abundant integral membrane protein in red cells[2,20,22]. Red cell phenotypes caused by a total deficiency of AE1 due to natural *AE1* mutations in cattle[11] and in humans[17], or due to genetic manipulations in

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mice[18], have demonstrated that incorporation of AE1 into the membrane skeletal network is essential for maintaining mechanical stability of the red cell membrane and the red cell shape, although AE1 is dispensable in the formation of the membrane skeletal network itself.

In mature red cells, AE1 comprises three different populations in the plasma membrane: tetramers linked to the spectrin/actin membrane skeleton through association with ankyrin, dimers attached to the membrane skeleton through association with adducin, and freely mobile dimers[3,6]. The assembly of these AE1 oligomers at the plasma membrane occurs progressively in early- and late-stage erythroblasts in accordance with the formation of the membrane skeletal network during erythroid cell maturation[5]. Previous studies have suggested that the interaction of AE1 with ankyrin is necessary for the cell surface delivery of newly synthesized AE1[9]. In murine erythroblasts, the synthesis of AE1 tetramers precedes that of dimers[10]. While newly synthesized AE1 tetramers are incorporated stably into the plasma membrane in early erythroblasts (probably in association with ankyrin), newly synthesized AE1 dimers appear rapidly in the plasma membrane of late erythroblasts and later are shuttled between the plasma membrane and microsomal compartments. On the other hand, it has been suggested that erythroid variants of chicken AE1 undergo endocytic recycling to the Golgi apparatus after the initial sorting to the plasma membrane[8]. Thus the precise mechanism for the trafficking of AE1 to the plasma membrane and its insertion into the membrane skeletal network remains unknown.

In this work, AE1 mutants with the murine polypeptide backbone and expressing cytoplasmic and extracellular tags for detection were prepared, in order to facilitate the analysis of their cell surface expression and internalization. The present study also reports that the insertion of the extracellular tag causes altered *N*-glycan processing of AE1. Biochemical analysis for clathrin-mediated endocytosis of the AE1 mutants described in the present study and several additional AE1 mutants will be published elsewhere[21].

Materials and Methods

Antibodies: The anti-murine AE1 polyclonal antibody (anti-AE1Ct) was prepared in rabbits against a synthetic peptide with the sequence of H_2N-CGLDEYDEVPMPV-COOH (the single letter abbreviations for amino acid residues are used throughout the present study), which corresponds to the C-terminal 12 amino acid residues and the additional N-terminal cysteine for conjugation with keyhole limpet hemocyanin. The antibody was purified on a Protein G-Sepharose 4FF column (Amersham). Other antibodies used were Cy3-labeled anti-FLAG M2 and anti-EEA1 (Sigma Chemical Co.), anti-GFP (Medical and Biological Laboratories, Nagoya, Japan), and anti-GM130 (BD Biosciences). Secondary antibodies labeled with Alexa-Fluor-405, -488 or -568 were from Molecular Probes.

Construction of cDNA clones: Murine wild-type AE1 (mAE1) cDNA (GenBank ID: NM_011403) was described previously[1,21]. The FLAG tag sequence (H_2N-DYKDDDDK-COOH) was introduced between R664 and G665 in the fourth extracellular loop of murine AE1 (Fig. 1) by inserting the FLAG epitope-coding DNA fragment into the *Xma* I site of the cDNA. The fragment was prepared by annealing oligonucleotides with the following sequences: 5'-CCGGATGGATTACAAGGATGACGACGATA AGGG-3' (sense) and 5'-CCGGCCCTTATCGTC GTCATCCTTGTAATCCAT-3' (antisense). This procedure resulted in the insertion of an extra methionine at the N-terminal end and an extra glycine and arginine at the C-terminal end, flanking the FLAG tag sequence (Fig. 1). The mAE1 cDNA and the FLAG-tagged murine AE1 cDNA (mAE1Flag) were subcloned into

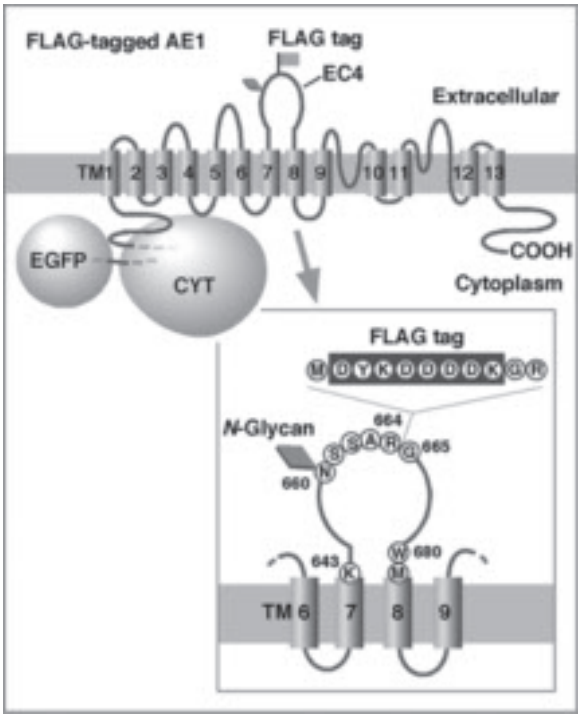


Fig. 1. Schematic illustration for the EGFP- and FLAG-tagged murine AE1 mutants. Mammalian AE1 consists of the cytoplasmic domain (*CYT*), transmembrane domain (*TM*), and a short cytoplasmic C-terminal tail. The EGFP-mAE1Flag contains EGFP and FLAG tags that were added to murine AE1 at the N-terminus and the C-terminal end of the *N*-glycosylation acceptor site (N660SS) in the fourth extracellular loop (*EC4*) (GenBank ID: NM_011403), respectively.

pEGFP-C1 (Clontech) using *Xho* I and *Hin* dIII sites in the vectors for expression of mAE1 with EGFP and murine AE1 with EGFP and FLAG tags (EGFP-mAE1 and EGFP-mAE1Flag, respectively).

Cell culture and expression of proteins: Cell culture and the transfection of HEK293 and COS-1 cells with plasmid vectors were carried out as previously described[12,19].

Analyses of proteins: Preparation of cell lysates, SDS-PAGE, immunoblotting, cell surface biotinylation, deglycosylation, densitometric quantification of immunoblotting signals and confocal laser microscopy were performed as described previously[1,12].

Analysis of internalization of cell-surface proteins: HEK293 or COS-1 cells grown on collagen-coated coverslips were washed with ice-cold phosphate-buffered saline (PBS). Cells were stained with the Cy3-conjugated anti-FLAG antibody at a 1:200 dilution at 4°C for 1 h and washed twice in ice-cold PBS. Cells were then incubated in the culture medium at 37°C for the indicated time periods to allow internalization, followed by fixation in methanol at −20°C.

Results

We previously reported that bovine erythroid AE1 tagged with an N-terminal EGFP showed plasma membrane expression in HEK293 cells, as observed for wild-type AE1[12]. To examine the effect of inserting the FLAG tag sequence into the fourth extracellular loop, the intracellular localization of mAE1Flag was compared to that of murine wild-type AE1 (mAE1) in HEK293 cells. Immunofluorescent staining with the anti-AE1Ct antibody demonstrated that mAE1Flag and mAE1 were predominantly distributed at the cell peripheries in permeabilized cells (Fig. 2A). The Cy3 signals indicating the FLAG tag was found only in the cells expressing mAE1Flag and co-localized with the signal for the C-terminus of AE1. On the other hand, no signal was detected when living cells transfected with mAE1Flag or mAE1 were stained with the anti-AE1Ct antibody. The Cy3 signal was detected at the periphery of live cells expressing mAE1Flag but not in the cells transfected with mAE1. Likewise, EGFP-tagged mAE1Flag localized to the plasma membrane, as shown by staining of the Cy3-anti-FLAG antibody at the cell surface (Fig. 2B). No signal was detected when cells expressing EGFP-mAE1Flag or EGFP-mAE1 were stained with the anti-AE1Ct antibody without permeabilization (data not shown). These results indicate that the FLAG- and EGFP-tagged murine AE1 constructs exhibit the correct membrane orientation and are properly transported to the cell surface.

When cells were incubated at 37°C after labeling with the Cy3-anti-FLAG antibody, rapid internalization of AE1 was observed, based on

the location of the Cy3 signal. After a 30-min incubation period, the vast majority of the Cy3 signal was detected in the cytoplasm within vesicular structures, some of which co-expressed EGFP (Fig. 2B). Cell surface expression and internalization of EGFP-mAE1Flag was also observed in COS-1 cells. Thirty minutes after incubation at 37°C, a population of the internalized EGFP-mAE1Flag showed co-localization with the early endosome and the Golgi apparatus, based on co-staining for organelle-specific markers (EEA1 and GM130, respectively) (Fig. 2C).

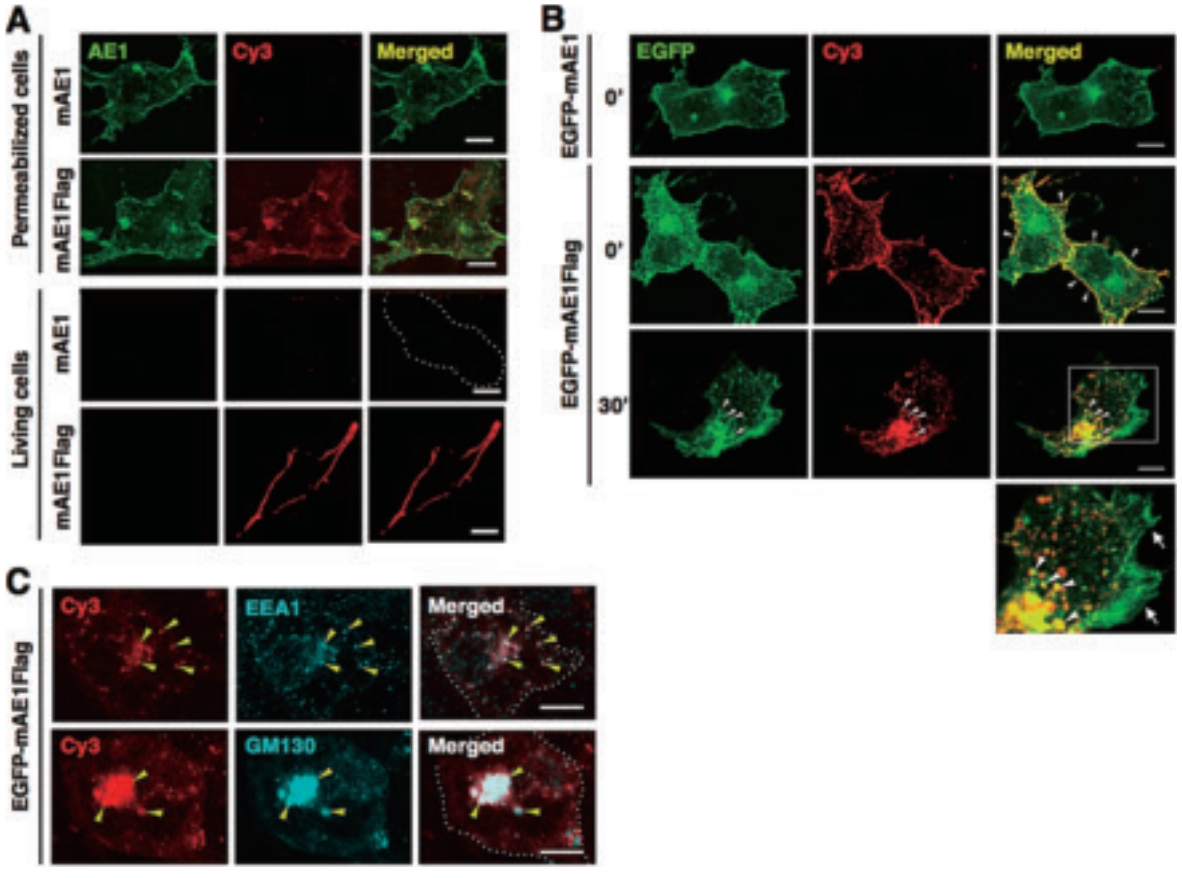


Fig. 2. Expression and intracellular localization of murine AE1 mutants in HEK293 or COS-1 cells. (A) HEK293 cells transfected with mAE1 or mAE1Flag were incubated for 48 h at 37°C. AE1 proteins were detected in cells fixed and permeabilized with methanol at −20°C (*Permeabilized cells*) or in living cells without permeabilization (*Living cells*). Cells were stained with the anti-AE1Ct (*AE1*) and the Cy3-anti-FLAG (*Cy3*) antibodies at 4°C for 1 h and then stained with Alexa Fluor 488-conjugated anti-rabbit IgG at 4°C for 30 min. Overlaid images of the signals for AE1 and FLAG are also shown (*Merged*). (B) HEK293 cells were transfected with EGFP-mAE1 or EGFP-mAE1Flag. Forty-eight hours after transfection, cells were stained with the Cy3-anti-FLAG antibody at 4°C for 1 h and immediately fixed with methanol at −20°C (*0'*), or further incubated at 37°C for 30 min after labeling with Cy3-anti-FLAG and then fixed (*30'*). EGFP- and FLAG-tag signals (*EGFP* and *Cy3*, respectively) partially co-localize at the cell surface in the cells fixed immediately after labeling with the Cy3-anti-FLAG (*0'*), and partially co-localize within certain intracellular compartments (but not at the cell surface) in the cells that were incubated at 37°C for 30 min after labeling for FLAG (*30'*). The indicated area in the merged image for the cell expressing EGFP-mAE1Flag is magnified and shown below. Areas of co-localization are indicated by arrowheads. The absence of the cell-surface Cy3 signal and the co-localization of the EGFP and Cy3 signals in intracellular compartments are shown by arrows and arrowheads, respectively. (C) EGFP-mAE1Flag expressed at the cell surface of transfected COS-1 cells was labeled with the Cy3-anti-FLAG antibody (*Cy3*) as described above, followed by a 30-min incubation at 37°C. Cells were fixed with methanol and incubated with anti-EEA1 (*EEA1*) or anti-GM130 (*GM130*) antibodies. Signals demonstrating co-localization of EGFP-mAE1Flag and EEA1 or GM130 are indicated by arrowheads. In some merged images in *A* and *C*, the peripheries of the cells are shown by broken lines. Bars, 10 μm.

Immunoblotting using several different antibodies demonstrated that EGFP-mAE1Flag consisted of a broad 155–165-kDa band and a sharp 144-kDa band (Fig. 3A). These polypeptides were slightly larger than those of EGFP-mAE1 (152–160 kDa and 142 kDa), presumably due to the size of the inserted FLAG tag sequence. Densitometric scanning of the signals showed that the larger 155–165-kDa polypeptide was predominant for EGFP-mAE1Flag [68% ± 2% (mean ± S. D.) of the total amount, n = 3], whereas the corresponding band was less abundant for EGFP-mAE1 [39% ± 4% (mean ± S. D.) of the total amount, n = 3]. Cell surface fractions of EGFP-mAE1Flag and EGFP-mAE1 obtained from cell lysates by cell surface biotinylation showed profiles very similar to those of total cell lysates (Fig. 3B). Deglycosylation studies demonstrated that the 144-kDa and 142-kDa bands described above represented AE1 polypeptides possessing endoglycosidase H (endo H)-sensitive immature *N*-linked oligosaccharide chains, while the larger bands (155–165 kDa and 152–160 kDa for EGFP-mAE1Flag and EGFP-mAE1, respectively) consisted of AE1 polypeptides carrying processed *N*-glycans resistant to endo H digestion (Fig. 3B). Consistent with these findings, AE1 polypeptides with endo

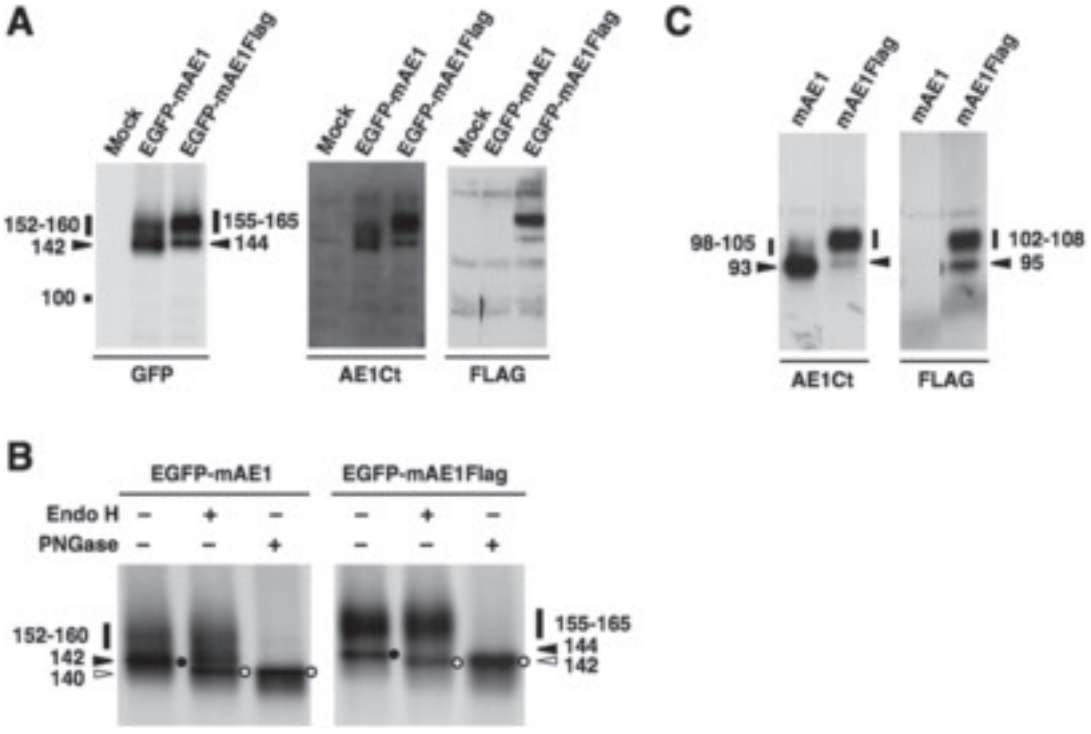


Fig. 3. Immunoblotting analysis for the effect of FLAG-tag insertion on the expression of AE1 mutants in transfected HEK293 cells. (A) Total cell lysates from HEK293 cells transfected with EGFP-mAE1 or EGFP-mAE1Flag were analyzed for protein expression of these AE1 mutants by immunoblotting using anti-GFP (*GFP*), anti-AE1Ct (*AE1Ct*), and anti-FLAG (*FLAG*) antibodies. *Mock* indicates the lysate from cells transfected with the empty vector. Migrating positions of two major polypeptides each for EGFP-mAE1 and EGFP-mAE1Flag are indicated in kDa. The migrating position of the 100-kDa marker is also shown. (B) Cell surface proteins in transfected HEK293 cells were biotinylated with NHS-SS-biotin (Thermo Fisher Scientific Inc.) and were separated from total cell lysates by using NutrAvidin beads (Thermo Fisher Scientific Inc.) as described previously[12]. The proteins were deglycosylated with endoglycosidase H (*Endo H*) or peptide-*N*-glycosidase F (*PNGase*), followed by the detection of AE1 mutants by immunoblotting with an anti-GFP antibody. *Bars* and *closed* and *open arrowheads* (and *circles*) indicate the migrating positions of AE1 polypeptides of which the *N*-glycan is endo H-resistant, endo H-sensitive, or deglycosylated, respectively. (C) Immunoblotting for mAE1 and mAE1Flag expressed in transfected HEK293 cells using anti-AE1Ct and anti-FLAG antibodies. Migrating positions of these mutants are indicated by bars and arrowheads, and molecular weights are indicated in kDa.

H-resistant *N*-linked oligosaccharides were predominant in the cells expressing mAE1Flag (approximately 85% of the total amount), whereas polypeptides with endo H-sensitive *N*-glycans were the major component in cells transfected with mAE1 (approximately 80% of the total) (Fig. 3C). The predominance of EGFP-mAE1Flag with processed *N*-glycans was also found in several additional mutants of this protein (data not shown). These data indicate that the addition of the extra FLAG tag sequence affected the processing of the *N*-glycans on AE1 polypeptides in the Golgi apparatus of transfected cells.

Discussion

The present study demonstrates that murine AE1 mutants with an artificial FLAG tag sequence (mAE1Flag and EGFP-mAE1Flag) are properly targeted to the plasma membrane in transfected HEK293 cells and their internalization can be traced by specific immunofluorescence labeling. The internalized EGFP-AE1Flag in the early endosomes appears to be recycled to the Golgi apparatus in transfected cells (Fig. 2). This is compatible with the previous observations that several erythroid variants of chicken AE1 undergo endocytic recycling to the Golgi after initial sorting to the cell surface[8]. However, the plasma membrane expression of EGFP-mAE1Flag (and mAE1Flag) that was transfected into the cells alone is contrary to previous findings that the cell surface delivery of newly synthesized murine AE1 required interaction of the AE1 with co-transfected ankyrin in HEK293 cells, which lack detectable ankyrin[9]. The presence or absence of the FLAG tag in transfected AE1 is not the simple cause for this discrepancy because the wild-type murine AE1 (mAE1) itself showed cell surface expression in this system (Fig. 2). Rather, it should be noted that the previous finding is based on the observation that pulse-labeled AE1 became endo H-resistant after a 4-h chase in HEK293 cells only when ankyrin was co-transfected[9]. The present study, however, demonstrates by several different approaches, including cell surface biotinylation and microscopic analysis, that several distinct AE1 mutants are sorted to the cell surface, and that a considerable population of EGFP-mAE1 in the cell surface fraction has endo H-sensitive immature *N*-linked oligosaccharides (Figs. 2 and 3). These data indicate that the processing of *N*-glycans, i.e., the conversion of the endo H-sensitive high-mannose form to the endo H-resistant complex oligosaccharide, does not necessarily occur before AE1 is transported to the cell surface. Cell surface delivery without *N*-glycan processing has similarly been demonstrated for human AE1 expressed in transfected HEK293 or COS-7 cells[15,16] and chicken erythroid AE1[8]. Thus, our data demonstrate that the interaction with ankyrin is not a prerequisite to the expression of murine AE1 and the EGFP- and/or FLAG-tagged mutants at the cell surface.

The cell surface expression without association to ankyrin and the internalization of EGFP-mAE1Flag may represent the recycling of AE1 dimers in murine late erythroblasts, as was suggested in a previous study[10]. Based on the temporal synthesis of AE1 oligomers in early- and late-stage erythroblasts, the authors proposed that in early erythroblasts, AE1 tetramers are transported to, and stably incorporated into, the plasma membrane, presumably in association with ankyrin. However, when ankyrin synthesis is downregulated in late erythroblasts, AE1 dimers are expressed at the cell surface but then recycled between the plasma membrane and intracellular compartments[10]. Their assumption fits the recent finding that the formation of the membrane skeletal network and the assembly of AE1 oligomers into the network occur progressively in early and late erythroblasts during erythroid cell maturation[5,13]. Therefore, the regulatory mechanism for the internalization of AE1 and its relevance to the association with ankyrin may play a key role in the insertion of

AE1 into the membrane skeletal architecture. The AE1 mutants described in this study would be suitable to explore such a mechanism. Although there was no evidence for dimer formation of murine AE1 mutants in this study, our previous studies demonstrated the formation of dimers for bovine AE1 in both *in vitro* and in transfected cells[12].

The present study also demonstrates that the insertion of the peptide including the FLAG sequence into the C-terminal side adjacent to the *N*-glycosylation acceptor site (N660, Fig. 1) can facilitate the processing of *N*-linked oligosaccharides (Fig. 3). It is interesting to note the possibility that the facilitated oligosaccharide processing is linked to the internalization and accumulation of FLAG-tagged AE1 at the Golgi. However, this is not likely the case because previous studies have shown that the processing of the oligosaccharide on human AE1 is dependent on its location: the *N*-glycan of AE1 at N642 (corresponding to N660 in murine AE1) is not processed efficiently in HEK293 cells as described above, while moving the *N*-glycosylation acceptor site to N555 in the preceding extracellular loop resulted in the conversion of the oligosaccharide into a complex form, and *N*-glycan processing at N555 and at N642 occurred independently in a single molecule[15,16]. 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[4,14]. Moreover, the *N*-glycan of human AE1 is normally processed to a complex form during erythroid cell maturation[7]. The present findings therefore indicate that the inserted hydrophilic fragment alters the steric hindrance surrounding N660, increasing its accessibility to the processing enzymes in HEK293 cells. Whether this structural alteration would affect AE1 functions such as the transport of anions or trafficking to the plasma membrane remains to be determined.

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